MYCOPLASMA AGASSIZII IN THE SONORAN POPULATION OF THE DESERT TORTOISE IN ARIZONA

by

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STATEMENT BY AUTHOR

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DEDICATION

This thesis is dedicated to my husband, Joe, who encouraged me to pursue my dream by providing unconditional support during the endless phases of this endeavor; and to my father and mother who taught me that nature is wondrous and worthy of study.

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ABSTRACT

Upper Respiratory Tract Disease (URTD), caused by the pathogens *Mycoplasma agassizii* and *M. testudineum*, has been documented in the desert tortoise (*Gopherus agassizii*). Although URTD was identified as the putative agent that led to federal listing of the Mojave population of the desert tortoise, little is known about this disease in the Sonoran population of the desert tortoise. The purpose of this study was to determine: 1) the prevalence of URTD across an urban gradient in Greater Tucson, Arizona, 2) the relationship between URTD and captive and free-ranging tortoises in Mohave, Maricopa, and Pima counties in Arizona, and 3) the effects of URTD on desert tortoise home range size and winter temperature selection. To determine the prevalence of *M. agassizii*, I used enzyme-linked immunosorbent assay (ELISA) to detect anti-*M. agassizii* antibodies in plasma samples, indicating previous exposure, and polymerase chain reaction (PCR) to detect *M. agassizii* DNA in nasal flush samples, indicating a current infection. I found that tortoises from suburban sites are 2.3 times more likely to test seropositive for antibodies to *M. agassizii* than tortoises from other sites in the Greater Tucson area. When I compared the seropositivity between captive and free-ranging desert tortoises from high-visitor-use site tortoises in Mohave, Maricopa, and Pima counties, I found that captive desert tortoises are 1.8 times more likely to test ELISA-positive than free-ranging desert tortoises, and desert tortoises in Pima County are 5.4 times more likely to test positive for anti-*M. agassizii* antibodies. When examining the effects of URTD on behavior, I found no significant difference between seropositive and seronegative tortoises for home range size, and the two clinically ill desert tortoises exhibited daily

activity during winter that resulted in increased environmental temperatures. Although additional study is needed to better understand the dynamics of *M. agassizii* in desert tortoise populations, our results are consistent with monitoring results that suggest that *M. agassizii* is widespread among tortoises in the Sonoran Desert and provides additional indirect evidence that captive tortoises are likely an important reservoir of URTD and may pass this disease onto free-ranging tortoises.

INTRODUCTION

Explanation of Problem

Over the past three decades, the desert tortoise (*Gopherus agassizii*) has experienced dramatic declines in some populations in the Mojave and Colorado Deserts (USFWS, 1994; Berry, 1997). These declines have been attributed to the cumulative impacts of human activities, predation, habitat loss and degradation, and disease (USFWS, 1994). In 1988, Desert tortoises with upper respiratory tract disease (URTD) were found in the Desert Tortoise Natural Area (DTNA; Kern County, California, USA; Jacobson et al., 1991); in 1989 43% of these tortoises showed clinical signs of this disease (Knowles, 1989; Brown et al., 1999a). Additional surveys through 1992 documented a 90% decline in the adult desert tortoise population (Berry, 1997). Upper respiratory tract disease was identified as a putative agent that led to this catastrophic decline. Since 1989, desert tortoises with URTD have been documented in populations of the Mojave desert tortoise in California, Nevada, and along the Utah-Arizona border (Jacobson, 1993; Berry, 1997; Lederle et al., 1997; Dickinson et al., 2002, 2005; Christopher et al., 2003, Berry et al., 2006). Largely because of this disease, the Mojave population of the desert tortoise (north and west of the Colorado River) was granted an emergency designation as endangered under the Endangered Species Act in 1989 (USFWS, 1989). Following subsequent surveys, the population was reclassified as threatened in 1990 (USFWS, 1990).

Literature Review

Upper respiratory tract disease has also been documented in gopher tortoises (*Gopherus polyphemus*) in Florida (USA; Brown et al., 1999b; Berish et al., 2000; McLaughlin et al., 2000; McCoy et al., 2007). On Sanibel Island, Florida, up to 50% of the adult gopher tortoises died after expressing clinical signs of URTD (McLaughlin, 1990). Berish et al. (2000) reported the presence of URTD in nearly one-third of tortoises sampled in a survey of 53 sites in Florida; all seropositive gopher tortoises were found in 14 sites located on public lands.

Mycoplasma agassizii was identified as a putative agent of URTD in Desert tortoises in 1994 (Brown et al., 1994) and in gopher tortoises in 1999 (Brown et al., 1999b). An additional mycoplasma (*M. testudineum*) was identified as a putative agent of URTD in desert tortoises in 2004 (Brown et al., 2004). Clinical signs of URTD include intermittent serous, mucoid, or purulent nasal discharge, ocular discharge, palpebral edema, conjunctivitis, and sunken eyes (Jacobson et al., 1991; Schumacher et al., 1993; Brown et al., 1994). This disease is highly contagious and transmitted by close contact between tortoises (Rosskopf, 1988; McLaughlin et al., 2000), and is often clinically silent and long-lasting; some tortoises have remained infected for up to a year (Schumacher et al., 1997). An enzyme linked immunosorbent assay is used to detect anti-*M. agassizii* antibodies in plasma, indicating previous exposure (Schumacher et al., 1993, 1997; Wendland et al., 2007); a polymerase chain reaction is used to detect *Mycoplasma* species-specific DNA, indicating a current infection (Brown et al., 1994; Brown et al., 1995).

Clinical signs of URTD have been observed since the 1970s in captive desert tortoises in California (Fowler, 1976; Rosskopf et al., 1981; Jacobson et al., 1991; Jacobson, 1993), and captive gopher tortoises in Florida (Brown et al., 1999b). Recent health assessments of captive desert tortoises in California found 82.7% from the Barstow area (Johnson et al., 2006), 61.8% from the Ridgecrest area, and 60% from the Palm Springs area (Berry et al., 2003) tested positive for exposure to *M. agassizii*. Ill captive tortoises are commonly returned to the wild due to the anxiety they generate in their custodians (Jacobson et al., 1995). The highest prevalence of URTD in free-ranging desert tortoises in California and Nevada, and gopher tortoises in Florida were found at sites where previous releases of captive tortoises occurred (USFWS, 1994; Jacobson et al., 1995; Berish et al., 2000; McLaughlin et al., 2000) which suggests that escaped or released captive tortoises may serve as disease vectors and pose a threat to healthy free-ranging populations.

Upper respiratory tract disease has been documented in the Sonoran desert tortoises in Arizona (Barrett, 1990; Barrett et al. 1990; Howland, 1994; AIDTT, 1996, 2000; Dickinson et al., 2002, 2005), with the percentage of tortoises testing seropositive higher closer to urban areas in Tucson (Johnson and Averill-Murray, 2004; Riedle and Averill-Murray, 2004; Jones et al., 2005, 2006). Thousands of desert tortoises have been held in captivity adjacent to some of the highest density wild populations reported in Arizona (Averill-Murray and Klug, 2000).

Although considerable research has been conducted on URTD, little information exists on how this disease affects home range size or winter temperature selection.

Researchers in the Sonoran Desert have observed symptomatic desert tortoises basking, foraging, and drinking during the traditional inactive season, November - March (S. Bailey, J. Jarchow, DVM, R. Repp, C. Schwalbe, E. Zylstra, unpubl. data). Investigations into ectothermic thermoregulation caused by infection have been conducted with lizards (Vaughn et al., 1974; Kluger et al., 1975; Kluger, 1978) as well as several turtle species (Monagas and Gatten, 1983; Swimmer, 2008). Monitoring the disease status and effects on desert tortoises throughout their range is important for understanding the dynamics of URTD in free-ranging populations (USFWS, 1994; Wendland et al., 2007).

Organization of the Thesis Format

The methods, results and conclusions of this study are presented in the three papers appended to this thesis, and the following chapter is a summary of the most important findings in these documents. All three of these studies are currently being prepared for submission. Each paper appears in the format for its particular journal of submission. Because the three studies are prepared separately for publication they each contain separate literature reviews relevant to the findings they present.

All manuscripts in this thesis are the result of research I conducted as a M. S. student at the University of Arizona. My major professor and committee members provided advice and guidance however, I was responsible for study design, data collection and analysis, and the presentation of results in this thesis. I am the senior author on all manuscripts resulting from my thesis research; coauthors include committee members who made contributions to this research.

PRESENT STUDY

Manuscripts in the appendices of this thesis are the result of research on the seroprevalence of *Mycoplasma agassizii* in Arizona's Sonoran population of the desert tortoise. The primary objective of this research was to determine if there was a relationship between the prevalence of *M. agassizii* in captive desert tortoises and free-ranging desert tortoises near urban areas in Arizona. This was investigated by first examining the relationship between *M. agassizii* across an urban gradient that compared seroprevalence of desert tortoises in captive, suburban, high-visitor-use, and remote locations in Greater Tucson, Arizona. A second study compared the seroprevalence of anti-*M. agassizii* antibodies in captive and free-ranging desert tortoises from high-visitor-use sites in Mohave, Maricopa, and Pima counties. In addition, we investigated the effects of URTD on home range size and temperature selection in desert tortoises in two sites in the Rincon Mountains, Pima County, Arizona.

I investigated the relationship between exposure to *M. agassizii* and an urban gradient by collecting tissue samples from captive desert tortoises from seven communities and from free-ranging desert tortoises in four suburban, four high-visitor-use and seven remote sites in Greater Tucson, Arizona. I found that the antibody prevalence was highest in suburban sites, with these tortoises 2.3 times more likely to test positive for exposure to *M. agassizii* than those in other tortoise site categories. That there were a high percentage of seropositive desert tortoises in all four site categories was a significant finding that suggests a potential link between urbanization and tortoise disease that I explore more fully in Appendices A and B.

I examined the relationship between *M. agassizii*, captivity and location, by collecting tissue samples from captive and free-ranging desert tortoises in high-visitor-use sites in Mohave, Maricopa and Pima counties. I found that seroprevalence varied by tortoise site category, with captive desert tortoises 1.8 times more likely to test ELISA-positive than free-ranging desert tortoises. I also found that ELISA results varied by location, with desert tortoises in Pima County 5.4 times more likely than those in Maricopa County to test positive for anti-*M. agassizii* antibodies.

The serologic results from my study do not demonstrate that captive tortoises are the original source of *M. agassizii*, but suggest that they may be an important reservoir of *M. agassizii* for the wild population, especially those in the urban-desert interface. No die-offs have been attributed to URTD in the Sonoran Desert, yet it is present across our sample populations, which may indicate that either natural anti-*M. agassizii* antibodies are present, or that *M. agassizii* is endemically stable in the Sonoran desert tortoise population in Arizona.

To determine the effect of *M. agassizii* on desert tortoise behavior, I used radiotelemetry to determine home range size for up to 22 tortoises, and temperature sensing data loggers to determine winter temperature selection for 12 tortoises at two sites in the Rincon Mountains, Pima County, Arizona. I examined the association between ELISA status and minimum convex polygon (MCP), 50% kernel and 95% kernel home range (KHR) size, and found no significant difference between seropositive and seronegative tortoises for 100% MCP, 95% KHR, or 50% KHR sizes. Additionally, I examined thermoregulatory behavior of asymptomatic and symptomatic desert tortoises

during the winter. The winter activity of two clinically ill desert tortoises resulted in daily increased carapace temperatures, suggesting that desert tortoises may seek environmental temperatures that induce a behavioral fever in response to an infection.

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APPENDIX A. PREVALENCE OF *MYCOPLASMA AGASSIZII* ACROSS AN URBAN GRADIENT IN GREATER TUCSON, ARIZONA. Draft manuscript to be submitted to the Journal of Herpetology: Jones, C. A., C. R. Schwalbe, and D. E. Swann.

LRH: C. A. Jones et al.

RRH: *M. agassizii* across an Urban Gradient

Prevalence of *Mycoplasma agassizii* across an Urban Gradient in Greater Tucson, Arizona

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Key Words: *Arizona, Desert Tortoise, Gopherus agassizii, Mycoplasma agassizii, Sonoran Desert, Upper Respiratory Tract Disease, URTD.*

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ABSTRACT

Upper respiratory tract disease (URTD), caused by the pathogens *Mycoplasma agassizii* and *M. testudineum*, poses a critical threat to the Mojave population of the Desert Tortoise (*Gopherus agassizii*). However, little is known about URTD in the Sonoran population of the Desert Tortoise. To determine the distribution of URTD in Greater Tucson, Arizona, USA we used enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *M. agassizii*, indicating previous exposure, and polymerase chain reaction

(PCR) to detect *Mycoplasma* species-specific DNA, indicating a current infection. From July 2002-May 2005, we collected blood and nasal lavage samples from 70 captive tortoises within Tucson and 152 free-ranging Desert Tortoises from 15 sites in 12 mountain ranges around Tucson. We used logistic regression to compare results from 1) captive, 2) suburban, 3) high-visitor-use, and 4) remote site tortoise populations to determine if there is an association between urbanization and prevalence of *M. agassizii*. Antibodies to *M. agassizii* were found in all four tortoise site categories. We found no difference between the odds of captive and free-ranging tortoises testing seropositive for *M. agassizii* and no difference between prevalence of antibodies in captive (44.3%), remote (42.9%), and high-visitor-use (40.4%) sites. However, the antibody prevalence was highest in suburban sites, where 63.3% of tortoises tested seropositive for exposure, and were 2.3 times more likely to test positive than at remote sites. Our results do not indicate whether captive tortoises are the original source of *M. agassizii*, but suggest that there may be a relationship between urbanization and this pathogen and that captive tortoises may be an important reservoir of *M. agassizii* in the urban-desert interface. No die-offs have been attributed to URTD in Arizona's Sonoran Desert, yet it appears to occur widely in Desert Tortoise populations throughout greater Tucson, Arizona.
Key Words: Arizona, Desert Tortoise, *Gopherus agassizii*, *Mycoplasma agassizii*, Sonoran Desert, Upper Respiratory Tract Disease.

INTRODUCTION

Over the past 30 years, the Desert Tortoise (*Gopherus agassizii*) has experienced dramatic declines in some populations in the Mojave and Colorado deserts (USFWS,

1994; Berry, 1997). These declines have been attributed to the cumulative impacts of human activities, predation, habitat loss and degradation, and disease (USFWS, 1994). In 1988, Desert Tortoises with upper respiratory tract disease (URTD) were found in the Desert Tortoise Natural Area (DTNA; Kern County, California, USA; Jacobson et al., 1991). In 1989, a detailed survey of the DTNA and nearby areas indicated that 43% of 468 Desert Tortoises presented clinical signs of this disease; 627 carcasses were recovered during this survey (Knowles, 1989). From 1989-1992, the adult Desert Tortoise population declined 90% within the DTNA (Berry, 1997). Upper respiratory tract disease was identified as a putative agent that led to this catastrophic decline. Largely because of this disease, the Mojave population of the Desert Tortoise (north and west of the Colorado River) was granted an emergency designation as endangered under the Endangered Species Act in 1989 (USFWS, 1989). Following subsequent surveys, the population was reclassified as threatened in 1990 (USFWS, 1990, 1994). In addition to the DTNA, URTD has been documented in the National Training Center, Fort Irwin, California; Las Vegas Valley and Yucca Mountain, Nevada; and the Beaver Dam Slope, along the Utah-Arizona border (Jacobson, 1993; Lederle et al., 1997; Schumacher et al., 1997; Dickinson et al., 2002, 2005; Berry et al., 2006). URTD has also been documented in Arizona's Sonoran Desert Tortoise population (Barrett, 1990; Barrett et al., 1990; Johnson and Averill-Murray, 2004; Riedle and Averill-Murray, 2004; Jones et al., 2008), though not in epidemic proportions (AIDTT, 1996a, 2000; Dickinson et al., 2002, 2005).

Upper respiratory tract disease has also been documented in Gopher Tortoises (*Gopherus polyphemus*) in Florida, USA (Brown et al., 1999b, Berish et al., 2000;

McLaughlin et al., 2000; McCoy et al., 2007). On Sanibel Island, Florida, up to 50% of the adult Gopher Tortoises died after expressing clinical signs of URTD (McLaughlin, 1990). Berish et al. (2000) reported the presence of URTD in nearly one-third of tortoises sampled in a survey of 53 sites in Florida; all seropositive Gopher Tortoises were found in 14 sites located on public lands. A recent survey of ten sites previously sampled for URTD in northern peninsular Florida found tortoises testing positive for antibodies to *M. agassizii* in areas that were previously seronegative; this suggests that URTD is more widespread than previously suspected (McCoy et al., 2007).

Mycoplasma agassizii was identified as a causative agent of URTD in Desert Tortoises in 1994 (Brown et al., 1994) and in Gopher Tortoises in 1999 (Brown et al., 1999b). In 2004, *M. testudineum* was also identified to cause URTD in Desert Tortoises (Brown et al. 2004). Clinical signs of URTD include intermittent serous, mucoid, or purulent nasal discharge, ocular discharge, palpebral edema, conjunctivitis, sunken eyes, and dullness of the skin and scutes (Jacobson et al., 1991; Schumacher et al., 1993; Brown et al., 1994). This disease is highly contagious and transmitted by close contact between tortoises (Rosskopf, 1988). *Mycoplasma* infections are often clinically silent and long-lasting; some tortoises have remained infected for up to a year (Schumacher et al., 1997).

Clinical signs of URTD have been observed in captive Desert Tortoises for many years in California (Fowler, 1976; Rosskopf et al., 1981; Jacobson et al., 1991; Jacobson, 1993), and captive Gopher Tortoises in Florida (Brown et al., 1999b). Recent health assessments of captive Desert Tortoises in California found 82.7% (148/179) from the

Barstow Area (Johnson et al., 2006), 61.8% (21/34) from Ridgecrest and Inyokern, and 60% (18/30) from Joshua Tree, Twentynine Palms and Palm Springs tested positive for exposure to *M. agassizii* (Berry et al., 2003). The highest prevalence of URTD in free-ranging Desert Tortoises in California and Nevada and Gopher Tortoises in Florida was found at sites where previous releases of captive tortoises occurred (USFWS, 1994; Jacobson et al., 1995; Berish et al., 2000; McLaughlin et al., 2000). Additionally, a higher prevalence of URTD has been reported near urban areas, which often have high concentrations of captive Desert Tortoises (USFWS, 1994; Berry et al., 2006; Jones et al., 2008). Ill captive tortoises are commonly returned to the wild due to the anxiety they generate in their custodians (Jacobson et al., 1995), suggesting that escaped or released captive tortoises may pose a threat as disease vectors to healthy free-ranging populations.

Thousands of Desert Tortoises are held in captivity adjacent to some of the highest density wild populations reported in Arizona (Averill-Murray and Klug, 2000). From 1981 to 2005, the Arizona-Sonora Desert Museum's Tortoise Adoption Program, sanctioned by the Arizona Game and Fish Department, adopted more than 2,500 tortoises into approved homes in the Tucson area, and it is likely that this is a small portion of the actual captive population. In addition to Desert Tortoises and Gopher Tortoises, URTD has also been observed in nonnative tortoise and turtle species commonly kept at pets, such as African spur-thighed (*Geochelone sulcata*), radiated (*G. radiate*), and leopard (*G. pardalis*) tortoises and Eastern box turtles (*Terrapene carolina*), and provide additional threats of introducing disease to free-ranging tortoises (Jacobson et al., 1991; Brown et al., 1999b; Wendland et al., 2007). On two separate occasions in 2000, African spur-

thighed tortoises were removed from the Tucson Mountains, Pima County, Arizona; both had only native vegetation in their fecal samples, which indicates that they had been living in that area for some time (M. Demlong, AGFD, unpubl. data).

Monitoring the disease status of Desert Tortoises throughout their range is considered important for understanding the dynamics of URTD in free-ranging populations (USFWS, 1994; Wendland et al., 2007). Little pathological information exists on either the captive or free-ranging Sonoran Desert Tortoise population (Jacobson et al., 1991; Dickinson et al., 2002, 2005). During 1990-4, a health study on free-ranging Sonoran tortoises was conducted at two remote sites (Harcuvar Mountains, La Paz County, and Little Shipp Wash, Yavapai County, Arizona; Dickinson et al., 2002; 2005). Although no clinical signs of URTD were observed in these remote populations, three out of 99 tortoises tested positive for exposure to *M. agassizii* or for a current URTD infection.

More recently, a preliminary disease study was conducted during 2001-2 at 10 Desert Tortoise study sites in Arizona. While no antibodies to *M. agassizii* were detected by an enzyme-linked immunosorbent assay (ELISA) in 80 samples from tortoises at eight remote sites (Bonanza Wash, La Paz County; Buck Mountains, Mohave County; East Bajada, Mohave County; Florence Military Reservation, Pinal County; Harcuvar Mountains, La Paz County; San Pedro Wash, Pinal County; Sugarloaf Mountain, Maricopa County; and West Silver Bells, Pima County), 53.5% (24/45) of tortoises in two high-visitor-use areas adjacent to Tucson, Arizona (Saguaro National Park East [SNPE] and Ragged Top Mountain) tested seropositive for antibodies to *M. agassizii*

(Johnson and Averill-Murray, 2004; Riedle and Averill-Murray, 2004). None of the SNPE tortoises were exhibiting clinical signs of URTD at the time sampling occurred, but observational records indicate that at least five tortoises at SNPE have exhibited clinical signs of URTD (nasal discharge, ocular discharge and palpebral edema) sporadically since 1999 (T. Esque and C. Schwalbe, U.S. Geological Survey, and D. Swann, National Park Service, unpublished data).

No study has examined the interaction between captive and free-ranging tortoises in the Sonoran Desert. The major objective of this study was to determine if there is an association between urbanization and exposure to *M. agassizii*. We used ELISA and polymerase chain reaction (PCR) analysis to determine the prevalence of URTD across an urban gradient that included captive Desert Tortoises and free-ranging Desert Tortoises from suburban, high-visitor-use, and remote locations in the vicinity of Tucson, Arizona. A secondary objective was to gather baseline data that would be invaluable for monitoring the health of Tucson area Desert Tortoises over time, especially for tortoise adoption programs, and for developing conservation programs for the wild populations of the Desert Tortoise.

MATERIALS AND METHODS

The Arizona-Sonora Desert Museum's Tortoise Adoption Program and members of the Tucson Herpetological Society facilitated access to captive Desert Tortoises from seven communities in metropolitan Tucson (Catalina, Catalina Foothills, Green Valley-Sahuarita, Marana, Oro Valley, Tucson, and Vail). We compiled a list of 321 individuals who adopted Desert Tortoises from 1996-2002, and then randomly selected two groups

of 100 to receive a letter requesting their participation in this study. The first 100 letters were mailed on 21 May 2003, a letter was mailed to an additional 100 custodians on 5 August 03. All custodians who contacted us as a result of the letter were included in the study.

Free-ranging Desert Tortoises were sampled from three site categories along an urban gradient. The site categories included 1) suburban, 2) high-visitor-use, and 3) remote sites. Suburban areas have limited access to hiking trails, are bordered by development that has been established for more than five years, and are known to be occupied by free-ranging Desert Tortoises. High-visitor-use areas are very easily accessed, popular with recreationists, have paved parking lots and multiple-use trails, and provide public programs that educate visitors about Desert Tortoises in the area. Remote areas lack easy public access, are reached by traveling on primitive dirt roads, and some are behind locked gates. In total, we sampled free-ranging Desert Tortoises from four suburban, four high-visitor-use, and seven remote sites in Pima and Pinal counties in the Greater Tucson area (Table 1).

During July 2002 through May 2005, we hand captured Desert Tortoises using standard methods (Murray and Schwalbe, 1997) following Arizona Interagency Desert Tortoise Team guidelines (AIDTT, 2000). To prevent transfer of pathogens between tortoises, we wore a fresh pair of disposable gloves for each tortoise and washed our hands and all equipment with veterinary disinfectant (chlorhexidine diacetate; AIDTT, 1996b) after processing each tortoise. With the exception of two occasions, we processed tortoises at the site of capture. On the two occasions, the tortoise was hand-carried from

its capture site to a central field processing location then returned to the point of capture within 1 hour. These tortoises were transferred in clean, cloth bags that were moistened to maintain temperature during transportation. We used hand-held Global Positioning System (GPS) units (Garmin E-map, GPS III-plus, Geko201; Olathe, KS) to determine the location of each tortoise encountered as Universal Transverse Mercators (UTMs), with CONUS NAD 27 as the datum.

Unmarked tortoises were marked using the notching system previously used at each site, with new numbers following those from the previous studies. At sites without an established numbering system, we marked tortoises using the standard notching system for Arizona (AIDTT, 2000). In addition to the notches, we also assigned each tortoise an identification number which was applied to the fifth vertebral scute with correction fluid and black ink, then covered with epoxy (Murray and Schwalbe, 1997) to facilitate easy identification if recaptured.

We examined each tortoise for clinical signs of URTD, shell anomalies, and parasites, and to determine sex (Murray and Schwalbe, 1997). We weighed tortoises with a 1, 5, or 10-kg spring scale and measured their midline carapace length (MCL) with pottery calipers to the nearest 1 mm (Christopher et al., 1997). Any evidence of harassment, injury, or predation by wild or domestic canids and felids on tortoises and evidence of released captive tortoises (i.e., those with paint on their carapace or a hole drilled in the marginal scutes) was photodocumented (Bjurlin and Bissonette, 2001; Zylstra, 2008). Additionally, we took photographs of the carapace, plastron, and nares of each tortoise, and archived them for future research (Berry, 1990).

We were prepared to rehydrate tortoises with a saline:dextrose (50:50) solution injected subcutaneously into the axillary region if they had an unusually low mass for body length, exhibited critical clinical signs of URTD, or voided excessively. We released each tortoise at the point of capture within 1 hour (AIDTT, 1996b).

Blood collected from each tortoise was used to run an ELISA that detects antibodies indicating previous exposure to *M. agassizii* (Schumacher et al., 1993, 1997). We manually restrained each tortoise on a pedestal (inverted coffee can) to immobilize them during processing. We cleaned the area where blood was to be sampled with diluted betadine followed by an alcohol swab (Berry and Goodlett, 2000). We collected <1 cc of blood with a syringe and 25 gauge 5/8" needle for ELISA analysis via subcarapacial or brachial venipuncture, and then applied pressure to the puncture site to prevent bleeding. Blood samples were immediately injected into a labeled Microtainer™ lithium heparin tube with plasma separator (#VT365958 VWR, West Chester, Pennsylvania), inverted gently 10-15 times, and placed on ice to prevent clotting (Jacobson et al., 1992; Berry and Goodlett, 2000). We centrifuged blood samples within 12 hours to separate the plasma. Plasma was transferred into a labeled 2-ml polypropylene cryogenic vial (#66021-944 VWR, West Chester, Pennsylvania), and stored at -20°C in a manual defrost freezer. The red blood cells were archived in the Microtainer™ lithium heparin tube for future population genetics work.

We performed a nasal lavage on each tortoise to collect samples for polymerase chain reaction (PCR) analysis to determine if the tortoise was currently infected with *M. agassizii* (Brown et al., 1994; Brown et al., 1995). Prior to collecting the nasal lavage, we

cleaned the head and gulars with an alcohol-soaked cotton ball, and allowed the skin to air dry. To conduct the nasal flush, we drew sterile saline (0.9% NaCl) into a 10-cc syringe with a 20 gauge needle (3 cc for all tortoises in 2002-3, 10 cc for tortoises ≥180 mm MCL and 6 cc for tortoises <180 mm MCL in 2004-5), then removed the needle from the syringe. In 2004-5, we then removed the needle stylet from either the winged 23 gauge x ¾" infusion set with tubing (#BD6253 VWR, West Chester, Pennsylvania) (2004) or 22 gauge x 1" SurFlo Teflon Resin I.V. Catheter (#14229-324 VWR, West Chester, Pennsylvania) (2005), and attached the plastic adapter with tubing or the catheter to the syringe without touching the distal tip.

To increase the volume returned during the nasal lavage, we modified our technique over the course of this study, and evolved from using only the syringe to deliver the saline in 2002-3, to attaching tubing in 2004 and a catheter in 2005 to the syringe to deliver the saline into each naris. Once the tortoise's head was stabilized, the distal tip of the syringe was placed against each naris in 2002-3, or the distal tip of the tubing (2004) or catheter (2005) was advanced 1-2 mm into the tortoise's naris, and one-half of the sterile saline was flushed into naris and caught in a sterile 4-ounce collection cup (#15173 VWR, West Chester, Pennsylvania). This procedure was repeated with the other naris with the remaining sterile saline. The nasal flush sample was transferred into a labeled 15-cc SuperClear polypropylene centrifuge tube (#21008-261 VWR, West Chester, Pennsylvania), placed on ice, then transferred to a -20 °C manual defrost freezer as soon as possible.

The Mycoplasma Research Laboratory, University of Florida (Gainesville) performed the ELISA and PCR diagnostic tests. We shipped the plasma and nasal flush samples overnight on dry ice to the lab at the end of each field season.

An ELISA was the most effective, rapid, and inexpensive method to detect the specific antibody in plasma or serum that would be present after exposure to *M. agassizii* (Schumacher et al., 1993; Wendland et al., 2007) during our study period. A positive result indicates that the tortoise has been previously exposed to *M. agassizii*. A negative result indicates that there are no detectable antibodies to *M. agassizii* in the plasma provided to the laboratory. A negative result does not mean that the tortoise will never develop the disease; it indicates that there are no antibodies present at the time the blood sample was taken. A suspect result indicates that the antibody level is intermediate between positive and negative, and is considered inconclusive.

Clinical signs may appear within one or two weeks post-exposure (Brown et al., 2002), but it takes six to eight weeks for an exposed tortoise to develop antibodies detectable by an ELISA (McLaughlin, 1997; Wendland et al., 2007). ELISA values are expressed as titers between the optical density of the plasma sample and that of a negative control. Sample titers <32 are negative, titers 32-64 are suspect, and titers >64 are positive for antibodies to *M. agassizii* (Schumacher et al., 1993; Wendland et al., 2007). This serologic technique only indicates that a tortoise has been exposed and immunologically reacted to *M. agassizii* and, therefore, cannot distinguish between asymptomatic carriers (which pose a threat to healthy tortoises) and tortoises that have

cleared the pathogen and are no longer infected (Brown et al., 1994; Schumacher et al., 1997).

Polymerase chain reaction analysis is designed to detect *M. agassizii* DNA based upon the amplification of the 16S ribosomal ribonucleic acid (rRNA) gene sequences in nasal secretions of Desert Tortoises (Brown et al., 1995). This technique, often used in conjunction with ELISA, detects the *M. agassizii* antigen. A positive result indicates that the tortoise is currently infected with *M. agassizii*. A negative result indicates the tortoise is not currently infected with the *M. agassizii*, or the mycoplasma organisms are in numbers too low to be detected by PCR.

We performed all statistical analyses with JMP software (Ver. 4.0; SAS Institute, Inc.). We used logistic regression analysis to determine if there was an association between tortoise site category and seropositive results. We then created indicator variables for the four tortoise site categories, using remote as a reference, and used logistic regression analysis to determine if there was a difference between the proportions of captive, suburban, high-visitor-use, and remote site category tortoises testing seropositive for exposure to *M. agassizii* as measured by ELISA (Zar, 1999; Ramsey and Shafer, 2002). Because ELISA-suspect results are inconclusive, tortoises testing serosuspect for exposure to *M. agassizii* were excluded from the analyses.

RESULTS

We collected a total of 222 blood and nasal flush samples from the indicated number of tortoises in the following site categories in Greater Tucson, Arizona: 70 captive, 49 suburban, 47 high-visitor-use, and 56 remote (Table 1). Tortoises ranged in

length from 131 to 330 mm MCL ($\bar{x}$ = 230.8 mm, *n* = 208, 95% CI = 226 to 236 mm) and in mass from 370 to 6300 g ($\bar{x}$ = 2284.2 g, *n* = 209, 95% CI = 2408 to 2559 g).

Antibodies to *M. agassizii* were present in 105 (47.3%) of the samples. Ninety-four (42.3%) tortoises were seronegative, and 23 (10.4%) were serosuspect (Table 1, Table 3). Of the 122 males, 66 (54.01%) were seropositive, 46 (37.7%) were seronegative, and 10 (8.2%) were serosuspect. Of the 82 females, 39 (47.6%) were seropositive, 34 (41.6%) seronegative, and 9 (11.0%) suspect. None of the 17 juvenile tortoises were seropositive, 13 (76.5%) were seronegative, and four (23.5%) were serosuspect.

Antibodies to *M. agassizii* were present in all seven captive tortoise communities and 13 of the 15 free-ranging tortoise sites sampled. The two free-ranging sites that did not test positive for exposure to *M. agassizii* were both remote sites with small sample sizes (Table 1). Seventy-four (52.5%) of the 141 adult free-ranging Desert Tortoises sampled tested ELISA-positive; 55 (39%) were seronegative, and 12 (8.5%) were serosuspect. All 11 juvenile free-ranging Desert Tortoises were seronegative. Of the 64 adult captive tortoises sampled, 31 (48.4%) were seropositive, 26 (40.6%) seronegative, and 7 (10.9%) serosuspect. Two (33.3%) of the six juvenile captive Desert Tortoises were seronegative, four (66.7%) were serosuspect.

Thirty-one (44.3%) of the captive tortoises, 31 (63.3%) of suburban, 19 (40.4%) of high-visitor-use, and 24 (42.9%) of remote site tortoises tested positive for exposure to *M. agassizii* (Table 1). ELISA results varied by tortoise site category, with tortoises at

suburban sites 2.3 times (95% CI from 1.03 to 5.40) more likely to test seropositive for exposure to *M. agassizii* than tortoises at remote sites (X_1^2=4.11, *p*=0.04; Table 2).

Only one of 222 nasal flush samples submitted tested positive for the presence of *M. agassizii* DNA based on the PCR amplification of the 16S rRNA gene. The remaining 221 results were negative, indicating that that was no correlation between the number of tortoises currently infected with *M. agassizii* (PCR) and tortoise site category.

Fifty-five (24.8%) of the 222 tortoises presented any clinical signs of URTD (Table 1); of these, 37 (67.3%) were seropositive, 14 (25.4%) seronegative, and 4 (7.3%) serosuspect. Twenty-six (70.3%) of these tortoises presented only ocular signs (palpebral edema, conjunctivitis, sunken eyes, ocular discharge), three (8.1%) presented only nasal signs (nasal discharge), and eight (21.6%) presented a combination of both ocular and nasal signs. Of the 167 asymptomatic tortoises, antibodies to *M. agassizii* were present in 68 (40.7%); 80 (36%) were seronegative, and 19 (11.4%) were serosuspect. The lone tortoise that tested PCR-positive was also seropositive, but asymptomatic.

Five tortoises (1 captive and 4 free-ranging) presented three to four (of 6 possible) clinical signs for URTD. Four of these tortoises were seropositive, one was serosuspect. Nine tortoises expressed two clinical signs, eight tested seropositive, one serosuspect. The remaining 41 tortoises expressed either one ocular or nasal clinical sign; 25 (61%) were seropositive, 13 (31.7%) seronegative, and three (7.3%) serosuspect.

Twenty-seven tortoises had evidence of previous URTD infection as indicated by depigmented nares or occluded nares.. Nineteen (70.4%) of these tortoise were

seropositive, five (18.5%) seronegative, and three (11.1%) serosuspect. The distribution of clinical signs across the four tortoise site categories is summarized in Table 3.

None of the 222 tortoises encountered expressed clinical signs for herpesvirus. Sixty (25.5%) tortoises showed some evidence of the shell disease cutaneous dyskeratosis, such as whitening of the scute seams, whitening between scales on the forelimbs, minor scute peeling, or pitting. Fifteen (6.4%) had one to four sand flies (*Lutzomyia tanyopsis*) on the head, limbs and / or carapace seams.

We documented 30 incidences of harassment by wild or domestic canids based on shell damage primarily on the marginal scutes above the limbs or the gular horns, 24 of these observed in free-ranging tortoises. Sixteen of these observations were at suburban sites, three at remote sites, and five at high-visitor-use sites. Fourteen tortoises were missing one or both gular horns. One tortoise was missing digits on its left hind limb; another was missing most of its left forelimb. Four tortoises had evidence of paired punctures on the plastron and carapace consistent with mountain lion bite. Only one free-ranging tortoise encountered had obvious evidence of previous captivity, the letter “D” painted on vertebral scutes 4-5.

DISCUSSION

Our study does not provide unambiguous evidence for an urban gradient of Desert Tortoise disease in the greater Tucson area, but suggests that there may be an urban effect associated with *M. agassizii*. The percentage of seropositivity was relatively high among all tortoise site categories and we found no differences among tortoises in captive, high-visitor-use, and remote sites. However, we did find a positive relationship between

proximity of tortoises to suburban sites and exposure to *M. agassizii* as measured by ELISA. Over 60% of tortoises in suburban sites tested seropositive; these tortoises were 2.3 times more likely to test positive than those sampled at remote sites. In addition, the only sites that yielded all seronegative results were remote sites, although the sample size for each was small (Table 1).

There are a number of potential explanations for this pattern, including the possibility that captive tortoise provide both a reservoir and a vector for URTD in the Tucson area. In our study, suburban site tortoises differed from other wild populations in that they were generally closer to urban neighborhoods and potentially more susceptible to anthropogenic influences such as free-roaming dogs, roads, and exposure to pet tortoises. All of these potential influences also exist in high-visitor-use sites, but most high-visitor-use sites were in protected natural areas where anthropogenic effects are presumably more limited.

Although the origins of *M. agassizii* remain unknown, there are many indirect lines of evidence that it could be an exotic pathogen. This evidence includes a history of observing clinical signs of URTD in captive Desert Tortoises (Fowler, 1976; Rosskopf et al., 1981; Jacobson et al., 1991; Jacobson, 1993), Gopher Tortoises in Florida (Brown et al., 1999b), and nonnative tortoise and turtle species commonly kept at pets (Wendland et al., 2007). In addition, seropositivity is very high in most captive populations (Berry et al., 2003; Johnson et al., 2006), and the highest prevalence of URTD in free-ranging Desert Tortoises in California and Nevada and Gopher Tortoises in Florida has been found at sites where previous releases of captive tortoises occurred (USFWS, 1994;

Jacobson et al., 1995; Berish et al., 2000; McLaughlin et al., 2000) and near urban areas where high concentrations of captive Desert Tortoises occur (USFWS, 1994). In our study, higher rates of seropositivity might be expected in contact areas between the captive and wild populations, such as occur in suburban sites.

Alternatively, it is possible that *M. agassizii* is a native pathogen and that higher rates of seropositivity are associated with environmental stressors of some kind. Hunter et al. (2008) recently found evidence that Desert Tortoises have natural antibodies to *M. agassizii* that can compromise the determination of infection status by ELISA, and outline methods that can distinguish natural antibodies from antibodies associated with infected tortoises. If *M. agassizii* is a natural pathogen, it is possible that higher seropositivity in suburban site tortoises may be related to stressors associated with proximity to urban areas, such as disruption of normal activities due to presence of free-roaming dogs, roads, or other anthropogenic impacts. For example, of the 24 tortoises with shell or limb damage that could be attributed to wild or domestic canids, two-thirds were at suburban sites, and we encountered packs of 3-5 presumably feral dogs at two suburban sites.

Greater Tucson represents a somewhat unique situation for Desert Tortoises in that the city and surrounding Pima County have both a very high concentration of captive tortoises and a large amount of both current and historic tortoise habitat that is occupied by humans at various densities. In a related study, Jones et al. (2008) compared health parameters of captive and wild populations in the Tucson area with other urban areas in Arizona, including Phoenix (Maricopa County) and Kingman (Mohave County). They

found that seropositivity was higher in captive tortoises in general (although not in Tucson), but that Desert Tortoises in Pima County were 5.4 times more likely to test ELISA-positive than in Maricopa County. In combination with our study, the results of these studies appear to be consistent with studies from the Mojave Desert (e.g., Jacobson et al. 1995, Berry et al. 2006, Johnson et al. 2006) that indicate some type of interaction between urbanization and tortoise disease, but it is unclear whether the effect is associated with captive tortoises, anthropogenic stress, or a combination of these and other unknown factors. Interestingly, no die-offs have been attributed to URTD in the Sonoran Desert, despite the fact that over 50% of the free-ranging tortoises in 13 of the 15 sites sampled in this study were positive for exposure to *M. agassizii*.

All juveniles included in this study were either seronegative (76.5%) or serosuspect (23.5%). URTD is likely fatal to smaller tortoises, so it is possible that the juvenile tortoises we encountered had not been exposed to *M. agassizii* (M. Brown, unpublished data). The four juvenile tortoises that tested serosuspect could have been in the early stages of URTD, but without retesting these results are inconclusive.

One-quarter (24.8%) of tortoises in this study were symptomatic, yet nearly one-half (47.3%) were seropositive. Of the 167 asymptomatic tortoises, 40.7% were seropositive. Clinical signs alone may not be a reliable tool to diagnose URTD because they may appear from two weeks to one year post-exposure to *M. agassizii*, but it takes six to eight weeks for an exposed tortoise to develop antibodies detectable by an ELISA (Schumacher et al., 1997; Brown et al., 2002). Some clinical signs, including nasal discharge and palpebral edema, can also be characteristic of other non-URTD health

conditions such as dehydration, poor nutrition, heat stress, infection of herpesvirus or another bacteria (*Chlamydia* or *Pasteurella*), or by recent ingestion of food or water (Schumacher et al.1997; McLaughlin et al., 2000; Johnson et al., 2006).

Polymerase chain reaction is less sensitive when tortoises are not expressing overt clinical signs of URTD (McLaughlin, 1997; Brown et al., 2002). Only 11 of the 222 tortoises sampled were expressing clinical signs that included nasal discharge at the time of nasal flush sampling; none of these tortoises were PCR-positive. Polymerase chain reaction results are highly dependent on the quality of the sample (Brown et al., 1999a). In Desert Tortoises, the mucosal surfaces of ventrolateral recesses in the nasal passage, the preferential site of bacterial growth, is not easily sampled by nasal flush, especially under field conditions (Schumacher et al., 1997). PCR-negative results could indicate that mycoplasma organisms were not present at time of sampling, or were present but in low numbers and the sampling technique failed to collect them.

Although the exact nature of the relationship between URTD and captive tortoises remains unknown, it is essential that management agencies continue to be active in taking steps to avoid spreading this disease into native populations. We recommend that, in translocation situations, all translocatee tortoises should be tested for natural and acquired antibodies as well as current clinical infections. Additional studies are needed to evaluate the impacts of other factors that affect natural population fluctuations like drought and malnutrition, on immune response and susceptibility. These may lead to improved knowledge based approaches to management of Desert Tortoise populations.

Continued monitoring in Arizona is a critical step towards determining the status of the Sonoran population of the Desert Tortoise, and including health evaluations that incorporate serology would be an efficient way to monitor the population's health over time. Upper respiratory tract disease may be subclinical, so determining the health status of a tortoise by physical examination alone may not be possible. Due to the difficulty of obtaining a quality nasal flush sample in the field for PCR analyses, unless the tortoise is expressing multiple clinical signs of URTD, future studies should focus their efforts on only collecting blood samples for serological analyses (i.e., ELISA or Western blot). Arizona is a rapidly growing state. As development continues to encroach upon Desert Tortoise habitat, more stringent guidelines for development and field research projects should be developed and implemented. These guidelines should also include health assessments with serology. Results can be used to monitor the Arizona tortoise population's health over time, and control the human spread of URTD when making translocation decisions.

Finally, the most effective way to control the human spread of URTD is through public outreach and education. Creative methods to inform the public to not collect or transport wild tortoises or release captive tortoises need to be developed and implemented. This can be achieved through a strategic educational program that uses posters, brochures, educational programs, and websites. The Tortoise Adoption Program provides a valuable alternative to euthanasia for displaced Desert Tortoises. Due to the potential to spread disease into naïve populations, a more rigorous educational component should be incorporated into each of the AGFD Tortoise Adoption Program

facilities to create more informed custodians. Prior to adopting a Desert Tortoise, in addition to completing a more stringent adoption application, potential custodians should attend an orientation that presents information on husbandry, while emphasizing the importance of keeping captive tortoises captive, and the potential impacts that URTD, or other diseases such as herpesvirus and pasteurella, can have on the wild population.

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Table 1. Sample sites, size (n), and gender (m = male, f = female, u = undetermined) from the four tortoise site categories, with ELISA (positive = E^+; negative = E^-; suspect = E^s) and positive clinical sign (CS^+) results. Captive sites include number of residences (in parentheses) sampled in each city, town, or community in Greater Tucson, Arizona, USA.

Tortoise Site Category	n	m	f	u	E^+	E^-	E^s	CS^+
Captive Residences in Greater Tucson								
Catalina (1)	2	2	0	0	2	0	0	1
Catalina Foothills (7)	12	5	7	0	6	5	1	1
Green Valley, Sahuarita (3)	4	3	1	0	2	2	0	0
Marana (9)	13	9	2	2	4	6	3	0
Oro Valley (4)	5	3	1	1	1	3	1	1
Tucson (24)	31	19	9	3	15	12	4	4
Vail (1)	3	3	0	0	1	0	2	1
Captive Tortoise Total	70	44	20	6	31	28	11	8
Suburban Sites								
Rincon Mts., Rocking K Development	18	9	9	0	13	3	2	8
Tortolita Mts., Saguaro Ranch Development	4	3	1	0	2	2	0	0
Tucson Mts., Panther Peak Wash, SNPW	19	10	8	1	10	8	1	8
Tumamoc Hill	8	3	4	1	6	2	0	4
Suburban Tortoise Total	49	25	22	2	31	15	3	20

Table 1. Continued

Tortoise Site Category	n	m	f	u	E^+	E^-	E^s	CS^+
High-Visitor-Use Sites								
Ragged Top, Sonoran Desert Monument	9	5	3	1	1	8	0	3
Rincon Mts., Mother's Day Fire, SNPE	25	12	10	3	13	8	4	9
Catalina Mts., Sabino Canyon Rec. Area	9	5	4	0	4	5	0	1
Tucson Mts., Visitor Center, SNPW	4	2	2	0	1	3	0	1
High-Visitor-Use Tortoise Total	47	24	19	4	19	24	4	14
Remote Sites								
Ninetysix Hills	13	6	5	2	7	5	1	0
Black Mountain	17	11	6	0	10	5	2	8
Desert Peak	1	1	0	0	0	1	0	0
Rincon Mts., Chiminea Creek	8	4	3	1	1	7	0	1
Sierrita Mts., Stevens Canyon	9	5	4	0	3	5	1	4
Suizo Mts.	5	2	3	0	3	1	1	2
Tortolita Mts., Derrio Canyon	3	0	1	2	0	3	0	0
Remote Tortoise Total	56	29	22	5	24	27	5	15
TOTAL Tortoise Samples	222	122	83	17	105	94	23	57

Table 2. Nominal logistic regression analysis of the association between tortoise site category and antibodies to *M. agassizii* in Desert Tortoises across an urban gradient in Greater Tucson, Arizona, USA.

						OR ($CI_{95\%}$)[c]	
Parameter	Estimate	SE	X^{2a}	*P*	OR[b,c]	Minimum	Maximum
Intercept	-0.12	0.28					
High-visitor-use	-0.11	0.42	0.08	0.78	0.89	0.39	2.01
Suburban	0.84	0.42	4.11	0.04	2.32	1.03	5.40
Captive	0.22	0.38	0.33	0.57	0.97	0.59	2.65

[a] Likelihood Ratio Test

[b] OR = odds ratio in relation to the remote tortoise site category

[c] Comparison of ELISA results: odds = odds ratio for positive ELISA result (odds E+/odds E-)

Table 3. ELISA serology results for all samples, and number of clinical signs of URTD in each tortoise site category sampled.

	No. Clinical Signs			
	1	2	3	4
ELISA results (n = 55)				
Seropositive	25	8	3	1
Seronegative	13	0	0	0
Suspect	3	1	1	0
Tortoise Site Category				
Captive (*n* = 13)	6	2	1	0
Suburban (*n* = 19)	13	4	1	1
High-visitor-use (*n* = 12)	10	1	1	0
Remote (*n* = 15)	11	3	1	0

APPENDIX B. SEROPREVALENCE OF *MYCOPLASMA AGASSIZII* IN CAPTIVE AND FREE-RANGING DESERT TORTOISES IN ARIZONA. Draft manuscript to be submitted to Journal of Wildlife Diseases. Jones, C. A., C. R. Schwalbe, and D. E. Swann.

LRH: Jones et al.

RRH: *M. agassizii* in captive vs. free-ranging tortoises in Arizona

SEROPREVALENCE OF *MYCOPLASMA AGASSIZII* IN CAPTIVE AND FREE-RANGING DESERT TORTOISES IN ARIZONA

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ABSTRACT

We studied the seroprevalence of upper respiratory tract disease (URTD) in free-ranging desert tortoises in high-visitor-use recreational areas, and in captive desert tortoises from nearby urban centers in Mohave, Maricopa, and Pima counties, Arizona. We used enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *Mycoplasma agassizii* and *M. testudineum*, the causative agents of URTD, and polymerase chain reaction (PCR) to detect *Mycoplasma* species-specific DNA, indicating a current URTD infection. Seroprevalence of antibodies to *M. agassizii* varied by tortoise category; captive desert tortoises were 1.8 times more likely to test ELISA-positive than free-

ranging desert tortoises. Enzyme-linked immunosorbent assay results also varied by location, with desert tortoises in Pima County 5.4 times more likely than those in Maricopa County to test positive for anti-*M. agassizii* antibodies. All PCR-positive results (n = 10) were from captive tortoises held in close proximity to other tortoises. Although additional study is needed to better understand the dynamics of *M. agassizii* in desert tortoise populations, our results provide additional indirect evidence that captive tortoises are likely an important reservoir of URTD and may pass this disease onto free-ranging tortoises.

Key words: Arizona, desert tortoise, enzyme-linked immunosorbent assay, *Gopherus agassizii*, *Mycoplasma agassizii*, Sonoran Desert, upper respiratory tract disease.

INTRODUCTION

The desert tortoise (*Gopherus agassizii*) has experienced dramatic declines, attributed to habitat loss and degradation, predation, collection, and disease in some populations in the Mojave and Colorado deserts (USFWS, 1994; Berry, 1997; USFWS, 2008). Studies on desert tortoises in the Mojave Desert noted catastrophic declines from 1988 to 1992 that were attributed to upper respiratory tract disease (URTD) (Jacobson et al., 1991; Berry, 1997). In 1989, largely because of the high mortality from respiratory disease, the Mojave population of the desert tortoise (north and west of the Colorado River) was emergency listed as endangered under the Endangered Species Act (USFWS, 1989); the population was listed as threatened in 1990 (USFWS, 1990). In Arizona, the Sonoran population of the desert tortoise is a Species of Greatest Conservation Need (AGFD, 2006), and protected under Arizona Revised Statues Title 17, under which it has

been unlawful to collect this species since 1989. The bacterium *Mycoplasma agassizii* was found to be the causative agent of URTD in desert tortoises in 1994 (Brown et al., 1994), and in gopher tortoises (*Gopherus polyphemus*) in 1999 (Brown et al., 1999b). An additional mycoplasma, *M. testudineum*, was identified as a putative agent of URTD in desert tortoises in 2004 (Brown et al., 2004).

Clinical signs of URTD include nasal discharge, ocular discharge, and palpebral edema, and were first documented in free-ranging desert tortoises in 1988 at the Desert Tortoise Natural Area in the western Mojave Desert, California (Knowles, 1989; Berry, 1990), and in gopher tortoises at Sanibel Island, Florida in 1989 (McLaughlin, 1990). Since 1988, URTD has been documented in Mojave desert tortoises in California, Nevada, and along the Utah-Arizona border (Jacobson, 1993; Berry, 1997; Lederle et al., 1997; Schumacher et al., 1997; Dickinson et al., 2002; Christopher et al., 2003; Dickenson et al., 2005). Upper respiratory tract disease has also been documented in the Sonoran population of the desert tortoise in Arizona (Barrett, 1990; Barrett et al., 1990; Howland, 1994; AIDTT, 1996a; Dickinson, et al., 2002, 2005), with the percentage of tortoises testing positive for exposure to *M. agassizii* being highest closest to urban areas around Tucson (Johnson and Averill-Murray, 2004; Riedle and Averill-Murray, 2004; Jones et al., 2008).

Clinical signs of URTD have been observed in captive desert tortoises in California (Fowler, 1980; Rosskopf et al., 1981; Jacobson et al., 1991; Jacobson, 1993) and captive gopher tortoises in Florida (Brown et al., 1999b) since the 1970s, long before the disease was documented in free-ranging tortoise populations. In California, recent

studies on captive desert tortoises found 82.7% (148/179) from the Barstow Area (Johnson et al., 2006), 61.8% (21/34) from Ridgecrest and Inyokern, and 60% (18/30) from Joshua Tree, Twentynine Palms and Palm Springs (Berry et al., 2003) tested positive for exposure to *M. agassizii*. The highest prevalence of URTD in free-ranging desert tortoises in the Mojave Desert and gopher tortoises in Florida were at sites where previous releases of captive tortoises occurred (USFWS, 1994; Jacobson et al., 1995; Berish et al., 2000; McLaughlin et al., 2000). Ill captive tortoises are commonly returned to the wild due to the anxiety they generate in their custodians (Jacobson et al., 1995), which suggests that escaped or released captive tortoises may serve as disease vectors and pose a threat to healthy free-ranging populations.

A higher prevalence of URTD has been reported near urban areas, which often have high concentrations of captive desert tortoises (USFWS, 1994; Berry et al., 2006; Jones et al., 2008). This has raised concern that the captive desert tortoises held adjacent to wild populations may be the source of *M. agassizii* for these free-ranging desert tortoises. The goal of this study was to assist conservation management of the desert tortoise in Arizona, a state where many residents keep captive tortoises as pets, by exploring the relationship between diseases in captive and wild populations of this species. We evaluated the health of free-ranging desert tortoises in high-visitor-use areas near three major urban centers, as well as captive desert tortoises from within each area. We collected plasma samples for enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *M. agassizii*, indicating previous exposure, and nasal flush samples for polymerase chain reaction (PCR) to detect *Mycoplasma* species-specific DNA, indicating

a current infection, and compared the percentage of seropositive captive and high-visitor-use area desert tortoises to determine the relationship between exposure to *M. agassizii* and captivity and location.

MATERIALS AND METHODS

From July 2002 to May 2005, we conducted health evaluations of free-ranging desert tortoises from high-visitor-use areas in Mohave, Maricopa and Pima counties, Arizona, and captive desert tortoises in Kingman, Phoenix, and Tucson, each county's primary urban center, in Arizona (Table 1, Figure 1). We defined high-visitor-use areas as those that were very easily accessed, popular with recreationists, and included either scheduled interpretive tours or signage that described desert tortoises of the area.

The Arizona-Sonora Desert Museum, Arizona Game and Fish Department (AGFD), National Turtle and Tortoise Society, and Tucson Herpetological Society facilitated access to captive desert tortoises from privately owned homes or state-sanctioned adoption facilities. All desert tortoise custodians were informed of the purpose of this study, clinical signs that indicate URTD, and potentially lethal effects that escaped or released captive desert tortoises can have on wild populations.

We hand captured desert tortoises using standard methods (Murray and Schwalbe, 1997) following Arizona Interagency Desert Tortoise Team guidelines (AIDTT, 2000). All tortoises were processed at the site of capture. We assigned each free-ranging tortoise a unique number that was permanently notched into marginal scutes with a triangular file following the standard notching system for Arizona (AIDTT, 2000) modified from Cagle (1939). In addition to notches, we also assigned each tortoise an identification number

which was applied to the fifth vertebral scute with white correction fluid and black ink, then covered with gel epoxy to facilitate easy identification throughout the study (Murray and Schwalbe, 1997). We used hand-held Global Positioning System units (Garmin E-map, GPS III-plus, Geko201; Olathe, KS) to determine the location of each tortoise encountered as Universal Transverse Mercators (UTMs), with NAD 27 CONUS as the datum.

We examined each tortoise for clinical signs of URTD (nasal discharge, ocular discharge, palpebral edema, and conjunctivitis), shell anomalies, and parasites, and to determine sex (Murray and Schwalbe, 1997). We weighed tortoises with a 1, 5, or 10-kg spring scale and measured their midline carapace length (MCL) with pottery calipers and a metal ruler to the nearest 1 mm (Christopher et al., 1997). To prevent transfer of pathogens between tortoises, we wore fresh examination gloves for each tortoise and washed our hands and all equipment with veterinary disinfectant (chlorhexidine diacetate; AIDTT, 1996b) after processing each tortoise.

We documented evidence of harassment, injury, or predation by wild or domestic canids on tortoises and evidence of previous captivity (i.e., those with paint on their carapace or a hole drilled in the marginal scutes; Bjurlin and Bissonette, 2001; Zylstra, 2008a). Additionally, we photodocumented the carapace, plastron, and nares of each tortoise, and archived them for future research (Berry, 1990).

We collected ≤1 cc of blood from each tortoise via brachial or subcarapacial venipuncture (as described in Jones et al., 2008). Samples were stored in a lithium heparin buffer on ice, and then centrifuged to separate the plasma within 12 hours. Both

plasma and red blood cells were stored in a -20˚C manual defrost freezer. Red blood cells were archived for future population genetics studies, and plasma was used to run an ELISA which detects the specific antibody that would be present after exposure to *M. agassizii* (as described in Schumacher et al., 1993; Wendland et al., 2007). A positive result (titer ≥64) indicates that the tortoise has been previously exposed to *M. agassizii*. A negative result (titer <32) indicates that there are no detectable antibodies to *M. agassizii* in the plasma provided to the laboratory. A negative result does not mean that the tortoise will never develop the disease; it indicates only that there are no antibodies present at the time the blood sample was taken. A suspect result (titer 32-64) indicates that the antibody level is intermediate between positive and negative, and is considered inconclusive without retesting. This serologic technique only indicates that a tortoise has been exposed and immunologically reacted to *M. agassizii* and, therefore, cannot distinguish between asymptomatic carriers (which pose a threat to healthy tortoises) and tortoises that have cleared the pathogen and are no longer infected (Brown et al., 1994; Schumacher et al., 1997). Clinical signs may appear within one or two weeks post exposure, but it takes six to eight weeks for an exposed tortoise to develop antibodies detectable by an ELISA (Schumacher et al., 1997; McLaughlin et al., 2000; Wendland et al., 2007).

We collected nasal flush samples from 378 tortoises (as described in Jones et al., 2008) for PCR analysis. These samples were stored on ice, then transferred to a -20˚C manual defrost freezer. Polymerase chain reaction is designed to detect the presence of *Mycoplasma* species-specific DNA through amplification of the ribosomal ribonucleic acid (rRNA) gene sequences in nasal secretions of desert tortoises (as described in Brown

et al., 1995). A positive result indicates that the tortoise is currently infected with *Mycoplasma*. A negative result indicates the tortoise is not currently infected, or the mycoplasma bacteria are in numbers too low to be detected by PCR.

The Mycoplasma Research Laboratory, College of Veterinary Medicine, University of Florida (Gainesville) performed the ELISA and PCR diagnostic tests. We shipped the samples overnight on ice to the lab at the end of each field season.

We performed all statistical analyses with JMP software (Ver. 4.0; SAS Institute, Inc.). We used logistic regression analysis to determine if there was an association between exposure to *M. agassizii* and captivity and location. We created indicator variables for tortoise categories and location, using captive tortoises and Maricopa County as a reference; then used logistic regression analysis to determine if there was a difference between the proportions of captive and free-ranging desert tortoises in each county testing positive for exposure to *M. agassizii* as measured by ELISA (Zar, 1999; Ramsey and Shafer, 2002). All confidence intervals presented are 95%. Because ELISA-suspect results are inconclusive, tortoises testing serosuspect for exposure to *M. agassizii* were excluded from the analyses.

RESULTS

We collected a total of 243 blood samples from captive desert tortoises in Kingman (n = 51), Phoenix (n = 122), and Tucson (n = 70); and a total of 159 blood samples from free-ranging desert tortoises in high-visitor-use areas in Mohave County (n = 24), Maricopa County (n = 88) and Pima County (n = 47; Table 1).

Sixty-one (25.1%) of the captive desert tortoise plasma samples submitted tested ELISA-positive for antibodies to *M. agassizii*; 164 (67.5%) were seronegative, and 18 (7.4%) were serosuspect. Twenty-nine (18.2%) of the plasma samples submitted from high-visitor-use area tortoises were seropositive; 125 (78.6%) were seronegative, and 5 (3.2%) were serosuspect for exposure to *M. agassizii*.

In Mohave County, eight (15.7%) of the captive, and one (4.2%) of the free-ranging desert tortoises tested positive for exposure to *M. agassizii*. Twenty-two (18%) of the captive, and nine (10%) of free-ranging desert tortoises in Maricopa County were seropositive. Thirty-one (44.3%) of captive and 19 (40.4%) of free-ranging desert tortoises in Pima County tested positive for anti-*Mycoplasma* antibodies (Table 1).

Enzyme-linked immunosorbent assay results varied by category, with captive desert tortoises 1.8 times (1.09<OR>3.21) more likely to test seropositive than free-ranging desert tortoises (X_1^2 =5.19, *P*=0.02). Seroprevalence of antibodies to *M. agassizii* also varied by location, with desert tortoises in Pima County 5.4 times (3.1<OR>9.4) more likely to test ELISA-positive than those in Maricopa County (X_2^2 =46.6, *P*<0.0001; Table 2).

We collected 219 nasal flush samples from captive desert tortoises in Kingman (*n* = 51), Phoenix (*n* = 93), and Tucson (*n* = 70); and 159 nasal flush samples from free-ranging desert tortoises in high-visitor-use areas in Mohave County (*n* = 24), Maricopa County (*n* = 88) and Pima County (*n* = 47). Ten of the nasal flush samples submitted tested PCR-positive for a current mycoplasma infection; all were captive tortoises awaiting adoption at two holding facilities in Phoenix. Seven were PCR-positive for the

M. agassizii DNA fingerprint, two for *M. testudineum*, and one for an unknown *Mycoplasma* sp. Three of these captive desert tortoises also tested positive for exposure to *M. agassizii* and had high ELISA titers (256) indicating either a recent or chronic infection (Wendland et al., 2007); two were symptomatic. Six tortoises were ELISA-negative and asymptomatic, and one was ELISA-suspect and symptomatic. Twenty-two of the desert tortoises sampled for PCR (18 captive, 4 free-ranging) were expressing clinical signs that included a clear or cloudy nasal discharge when sampling occurred; only three of these tortoises were PCR-positive.

We documented 35 incidences of harassment by wild or domestic canids based on shell damage primarily on the marginal scutes above the limbs or the gular horns, 17 of those involved free-ranging tortoises. Twelve tortoises were missing one or both gular horns. The study sites where the damage was most severe (as indicated by missing gular or multiple marginal scutes) were those closest in proximity to urban development (Golden Shores and Silver Creek Rd in Mohave County; McDowell Mountain, San Tan Mountain, and South Mountain in Maricopa County; and Sabino Canyon in Pima County), or where we encountered packs of 3-5 presumably feral dogs (East Bajada in Mohave County; and Saguaro National Park West in Pima County). No free-ranging tortoises had evidence of previous captivity.

DISCUSSION

Our results suggest a number of patterns in prevalence of *M. agassizii* among captive and urban-wildland interface free-ranging desert tortoises in the Sonoran Desert that are consistent with previous studies in the Mojave Desert, but with some important

differences. Our finding that a higher percentage of captive desert tortoises tested positive than those in nearby wild populations is consistent with previous studies (Berry et al., 2003, 2006; Johnson et al., 2006). Twenty-five percent of total captive desert tortoises in our study had anti-*M. agassizii* antibodies; this is lower than results from two studies in California where 60% of tortoises sampled from Ridgecrest and Inyokern, 61.8% from Joshua Tree, Twentynine Palms and Palm Springs (Berry et al., 2003), and 82.7% the Barstow Area (Johnson et al., 2006) tested ELISA-positive.

Similarly, our percentage of seropositivity (18.2%) in free-ranging tortoises was lower than observed in the Mojave population of the desert tortoise, where 25-38% of 12 to 20 tortoises sampled from 1992-7 in the Desert Tortoise Natural Area, California (Brown et al., 1999a), 43% (122/283) of tortoises sampled from Yucca Mountain, Nevada (Lederle et al., 1997), and 50% (72/144) of tortoises sampled from Las Vegas Valley, Nevada (Schumacher et al., 1997), were seropositive. Percentage of seropositivity has also varied from 30 to 85% in studies of gopher tortoise populations at various sites in Florida (Beyer, 1993; Smith et al., 1998; Berish et al., 2000; Karlin, 2008). However, our ELISA-positive results for free-ranging tortoises are significantly higher than those from two previous health studies conducted in central Arizona. The first was conducted at two long-term monitoring plots in central Arizona (Harcuvar Mountain and Little Ship Wash) from1990-4, and <3% (3/101) of tortoises had anti-*Mycoplasma* antibodies or a current infection (Dickinson et al., 2002, 2005); the second was from 2001-2, where all tortoises sampled from six long-term monitoring plots (0/ 41) were ELISA-negative (Riedle and Averill-Murray, 2004).

Frequency of URTD occurrence in both free-ranging and captive tortoises in Pima County was significantly higher than in Maricopa and Mohave County. Indeed, one-half of the seropositive captive desert tortoises and 65% of the free-ranging tortoises sampled in our study were from Pima County. Two recent disease studies in Arizona found a higher prevalence of antibodies to *M. agassizii* in urban and remote areas in Tucson and elsewhere in this county. Jones et al. (2008) examined tortoise health across an urban gradient in and near Tucson and found that desert tortoises in the urban-desert interface had the highest percentage of seropositivity; however, seropositive tortoises were also found in remote locations. Another study found that 53% (24/45) of tortoises in three sites near Tucson were seropositive (Johnson and Averill-Murray, 2004; Riedle and Averill-Murray, 2004). This pattern of differences in seropositivity among populations, with the suggestion of an urban effect, also seems evident in desert tortoises in the Mojave Desert (Jacobson et al., 1995; Berry et al., 2006; Johnson et al., 2006) although further testing of non-urban areas would be important to confirm the pattern.

Although no previous study has investigated and compared seroprevalence of URTD in captive and free-ranging desert tortoises from the same geographic area, results from disease studies have shown that higher percentages of anti-*M. agassizii* antibodies occur in captive desert tortoises (Berry et al., 2003; Johnson et al., 2006) and free-ranging desert tortoises in the urban-desert interface (Jacobson et al., 1995; Berry et al., 2006) than in free-ranging desert tortoises in remote locations (Berry et al., 2006; Jones et al., 2008). The implication of previous studies is that because *M. agassizii* infects multiple turtle and tortoise species commonly held as pets in captivity (Jacobson et al., 1991;

Brown et al., 1999a, b), captive desert tortoises may acquire this pathogen and introduce it into nearby wild desert tortoises populations if they escape or are intentionally released (Jacobson et al., 1995; Johnson et al., 2006). Indirect support for this argument is based on the known history of URTD, which was first observed in captive desert tortoises and gopher tortoises in the 1970s (Fowler, 1980, E. Jacobson unpubl. data), and continues to be commonly treated by veterinarians (Jacobson et al., 1991; Jacobson and McLaughlin, 1997; J. Jarchow, DVM, unpubl. data). Although it remains unknown if *M. agassizii* is an exotic pathogen, the disease was not observed in free-ranging desert tortoises until 1988 or in free-ranging gopher tortoises until 1989.

Our results provide additional indirect support for the exotic pathogen argument, although there are other possible explanations. The higher prevalence of *M. agassizii* in Pima County may be associated with the very large captive desert tortoise population in Tucson, which is estimated to be over 10,000 tortoises (C. Schwalbe, unpubl. data). Tucson has a naturally high population of tortoises close to human populations (Swann et al., 2002; Jones et al., 2008; Zylstra, 2008b), and since the AGFD-sanctioned Tortoise Adoption Program began in 1982, up to 600 tortoises have been adopted into private homes in Kingman, 1,500 in Phoenix, and 2,500 in Tucson (S. Cate, AGFD; S. Goodman, AGFD; S. Poulin, ASDM; pers. comm., 2005). Because there are no special licenses to hold desert tortoises, or laws prohibiting propagation, there are no records to accurately reflect the captive population for Arizona. In this regard, Tucson may be similar to areas in California such as Barstow, which also has a history of wide-scale

adoption of desert tortoises as pets, and high prevalence of seropositivity in captive and nearby wild tortoise populations (Johnson et al., 2006).

However, in contrast to areas in California, the overall prevalence of *M. agassizii* is lower in Arizona, and there have been no recorded catastrophic die-offs attributed to URTD in the Sonoran Desert (AIDTT, 1996a, 2000; Dickinson et al., 2002, 2005; Jones et al., 2008). This could be due to a different rate of progression of *M. agassizii* into the wild population because of differences in human population growth or tortoise natural history or because desert tortoises in Arizona have natural antibodies that are effective against *M. agassizii* which cannot be distinguished from acquired antibodies when analyzed with ELISA (Hunter et al., 2008). It is also possible that environmental effects, such as extreme drought, that may interact with URTD to cause die-offs in the Mojave Desert do not occur or interact in the same way with the disease in the Sonoran Desert. Finally, it is possible that *M. agassizii* is native to Sonoran desert tortoises, which have developed effective antibodies that do not occur in Mojave desert tortoises. Because we only collected one sample from each tortoise, the seropositive results only provide a snap-shot of tortoise health for that date. Additional studies that implement repeated sampling over time would be needed to determine if a population has a stable proportion of seropositive tortoises, indicating an endemic disease, or increasing rate of seropositivity, indicating an acute infection (USFWS, 2008).

Brown et al. (1999a) found PCR to be less sensitive when tortoises are not overtly expressing clinical signs of URTD; findings corroborated in additional studies (McLaughlin, 1997; Brown et al., 2004). The high number of PCR-negative results in our

study could indicate that tortoises were not symptomatic, the mycoplasma organisms were not present at time of sampling, or were present but in low numbers and the sampling technique failed to collect them. In desert tortoises, the mucosal surfaces of ventrolateral recesses in the nasal passage, the preferential site of bacterial growth, is not easily sampled by nasal flush, especially under field conditions (Brown et al., 1999a). For this reason, we continued to improve our nasal flush technique and applied three methods of delivering the saline when flushing the nares. The method that delivered the highest number (five) of PCR-positive results utilized a 22 gauge x 1" SurFlo Teflon Resin I.V. Catheter (#14229-324 VWR, West Chester, Pennsylvania) attached to a 10-cc syringe, and advanced 1-2 mm into the tortoise's naris (as described in Jones et al., 2008).

However, all ten of the tortoises with active disease were captive tortoises that had been placed in a holding facility within two months of testing and were awaiting adoption in Phoenix, which further suggests a strong link between *M. agassizii* and captivity. In addition, over 80% (18 of 22) of the tortoises that presented clinical signs were captives. Both adoption facilities housed multiple tortoises per enclosure; one housed multiple tortoise species together including African spur-thighed tortoises (*Geochelone sulcata*), one of several nonnative tortoise species known to carry anti-*M. agassizii* antibodies (Jacobson, 2007). It is possible that these tortoises entered the facility with current infections. However, it is also possible that the cumulative effects of acclimating to a new location, being housed with multiple tortoises, and the close contact among tortoises may have induced stress that lowered these tortoises's immune systems, making them more susceptible to URTD (Jacobson et al., 1991). Because *M. agassizii* is

spread by close contact among tortoises, the relationship between this increased exposure and crowding is a potential alternative explanation for why captive tortoises may exhibit higher seropositivity than free-ranging tortoises. Additional studies on the effects of stressors on tortoise immune systems are warranted.

Because it takes six to eight weeks for an exposed tortoise to develop antibodies detectable by an ELISA (Wendland et al., 2007), the sample from the PCR-positive-ELISA-suspect tortoise may have been collected during this period. The PCR-positive sample with an unknown DNA fingerprint was *Mycoplasma* positive; however, the DNA fingerprint was not a *M. agassizii* isolate (*M. testudinis*, *M. testudineum* or *Acholeplasma laidlawii*), and was unrecognized by the Mycoplasma Research Lab (L. Wendland, University of Florida, pers. comm., 2005).

MANAGEMENT IMPLICATIONS

Considering that no die-offs attributed to URTD have been documented in the Sonoran Desert even with the widespread distribution and sometimes high prevalence of tortoises seropositive for antibodies to *Mycoplasma*, this disease appears not to be as much of a threat in the Sonoran desert tortoise population as it has been in the Mojave. Based on our results, and the cumulative ELISA-negative results of annual disease sampling on each of the long-term monitoring plots in Arizona since 2002 (AGFD, unpublished data), new studies on prevalence of URTD in the Sonoran population of the desert tortoise are not necessary at this time. Because this disease can be subclinical, and not easily detected though physical examination, we do, however, recommend that the sampling and testing of desert tortoises for mycoplasma or other potentially significant

diseases (i.e., herpesvirus, iridovirus) using serology (i.e., Western blot, ELISA) continue to be included in the statewide monitoring protocol for desert tortoises in Arizona (as described in Averill-Murray, 2000; AGFD unpublished data). These results can be used to continue to monitor the health of the Sonoran desert tortoise populations in Arizona over time, and to better understand the true impact of disease on population dynamics (e.g., reproduction, survival, size class effects). Due to the expense and unreliable detection of a mycoplasmal infection using PCR, we recommend that samples for microbiology (i.e., PCR, culture) only be collected from tortoises expressing multiple clinical signs of a current infection.

Because URTD has not been documented in all populations of desert tortoises in the Sonoran Desert, we also recommend that all translocatee tortoises be tested for natural and acquired antibodies and current clinical infections prior to any translocation that will relocate tortoises further than approximately the diameter of an average home range (14.3 ± 13.72 ha; Averill-Murray et al., 2002) for a Sonoran desert tortoise, or ≤500 m. When moving tortoises a shorter distance, out of harms way during development projects (as described in the AGFD's Guidelines for Handling Sonoran Desert Tortoises Encountered on Development Projects (http://www.azgfd.gov/pdfs/w_c/tortoise/Tortoise%20handling%20guidelines.pdf), disease testing may only be necessary if a tortoise expresses clinical signs of disease.

Mycoplasmal infections often remain subclinical until some other factor such as stress, environmental conditions, or other infectious agents, triggers progression to clinical disease. Because disease may be a secondary response to stressors, additional

studies that evaluate the impacts of other factors that affect natural population fluctuations (such as drought or malnutrition) on immune response and susceptibility may lead to improved knowledge-based approaches to management of desert tortoise populations. Understanding the dynamics of URTD spread in natural populations may provide insight into other emerging infectious diseases such as herpesvirus or iridovirus.

Because our data do suggest that captive desert tortoises may act as a reservoir of *M. agassizii* for free-ranging desert tortoises, and that captives may spread the disease into wild populations, it is important to emphasize how people can help control the spread of this and other diseases through public outreach and education. In Arizona, an individual may possess, transport (within Arizona), or give away a desert tortoise without a special license if that individual possessed it before April 28, 1989. Any individual who possesses a desert tortoise may propagate it under R12-4-404(B)(D) and R12-4-407(A)(1), and hold offspring in captivity for ≤24 months from the date of hatching. After 24 months have passed, the individual shall dispose of the offspring of desert tortoises by giving them as a gift or as directed in writing by the AGFD. In an effort to discourage captive propagation and spread of diseases, in January 2008 the AGFD developed more stringent Tortoise Adoption Program Guidelines that limit tortoise adoptions to one per household. Because the Tortoise Adoption Program provides an alternative to euthanasia for displaced tortoises, we recommend that the program incorporates a required educational component for potential custodians prior to adoption that provides information and evidence on effects of infectious diseases on wild tortoise populations. These and other educational programs should also emphasize that

unconfined dogs may kill or seriously harm wild tortoises (USFWS, 2008; Zylstra, 2008a) and encourage dog-owners to leash their dogs in natural areas at all times.

In addition, yard inspections should be conducted prior to adoptions to ensure that tortoise enclosures are designed to prevent escape. Additional information on the importance of keeping captive tortoises captive, as well as contact information for questions about tortoise care could be communicated to custodians in an annual mailer reminding them of their responsibilities.

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Table 1. Locality names, number of tortoises sampled, and gender (m = male, f = female, u = undetermined) from each site with ELISA (positive = E^+; negative = E^-; suspect=E^s) and positive clinical sign (CS^+) results.

Sample Site Location	n	m	f	u	E^+	E^-	E^s	CS^+
Mohave County								
Kingman Area Captive Tortoises	51	28	15	8	13	37	1	2
Free-Ranging Tortoises								
Silver Creek Rd, West of Black Mts.	6	4	2	0	0	6	0	1
Golden Shores, Oatman HWY	4	3	1	0	0	4	0	0
East Bajada, Black Mts.	9	4	2	3	0	9	0	0
Hualapai Mnts, Shingle Canyon	10	4	2	4	1	9	0	0
Maricopa County								
Phoenix Area Captive Tortoises	122	61	39	22	22	94	6	12
Free-Ranging Tortoises								
Buckeye Hills Regional Park	2	1	1	0	0	2	0	0
Cave Creek Regional Park	16	5	10	1	4	11	1	1
Desert Outdoor Center	4	1	2	1	0	4	0	0
Dreamy Draw Area	1	0	1	0	0	1	0	0
Estrella Mountain Regional Park	1	0	1	0	0	1	0	0

Table 1. Continued

Sample Site Location	n	m	f	u	E^+	E^-	E^s	CS^+
Lake Pleasant Regional Park	14	10	4	0	0	14	0	0
McDowell Mountain Regional Park	14	3	9	2	1	13	0	0
San Tan Mountain Regional Park	8	3	5	0	1	7	0	0
South Mountain Park/Preserve	4	0	4	0	0	4	0	0
Spur Cross Ranch Conservation Area	3	0	2	1	0	3	0	0
Sugarloaf Mountain, Tonto NF	16	3	13	0	0	16	0	0
Usery Mountain Regional Park	4	3	1	0	3	1	0	0
White Take Mountain Regional Park	1	0	1	0	0	1	0	0
Pima County								
Tucson Area Captive Tortoises	70	44	20	6	31	28	11	4
Free-Ranging Tortoises								
Ragged Top, Sonoran Desert Monument	9	5	3	1	1	8	0	0
Saguaro NP East, Rincon Mts.	25	12	10	3	13	8	4	1
Sabino Canyon Rec. Area, Catalina Mts.	9	5	4	0	4	5	0	1
Saguaro NP West, Tucson Mts	4	2	2	0	1	3	0	0

Table 2. Nominal logistic regression analysis of the association between location and tortoise category and antibodies to *M. agassizii* in captive and free-ranging desert tortoises Mohave, Maricopa, and Pima counties, Arizona, USA.

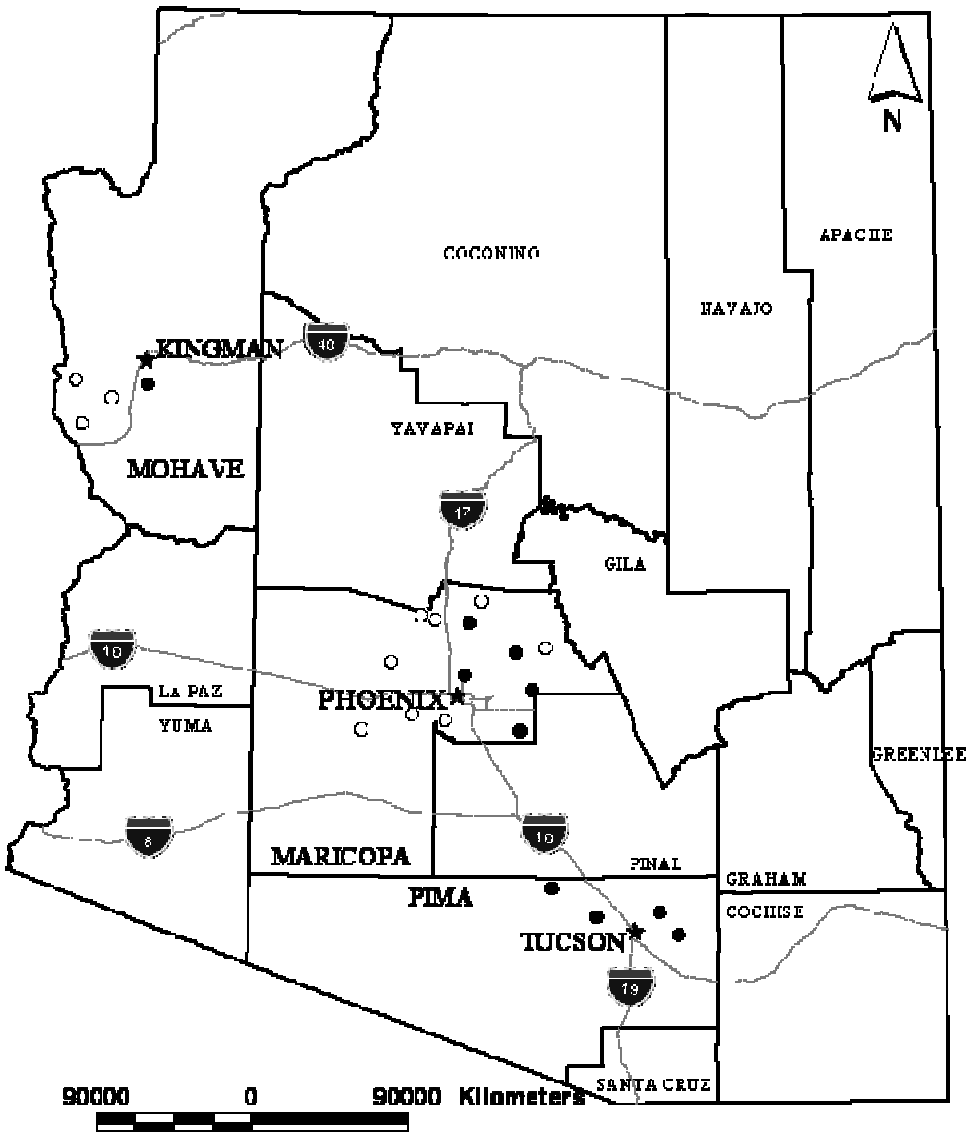
					OR ($CI_{95\%}$)[b]		
Parameter	Estimate	SE	X^{2a}	*P*	OR	Minimum	Maximum
Intercept	-2.10	0.27					
Captive	0.61	0.27	5.19	0.02	1.85 [c]	1.09	3.21
Pima Co.	1.68	0.28	38.03	<0.0001	5.39 [d]	3.13	9.44
Mohave Co.	-0.33	0.41	0.67	0.41	72.00 [d]	0.31	1.55

[a] Likelihood Ratio Test

[b] Comparison of ELISA results: odds = odds ratio for positive ELISA result (odds E+/odds E-)

[c] OR = odds ratio in relation to high-visitor-use site tortoises

[d] OR = odds ratio in relation to Maricopa County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LIST OF FIGURES

Figure 1. Locations of desert tortoise blood collection sites in Arizona. Circles indicate sites surveyed during this study. Closed circles are sites where seropositive tortoises were found; open circles are sites where only seronegative tortoises were found.

APPENDIX C. EFFECTS OF *MYCOPLASMA AGASSIZII* ON HOME RANGE SIZE OF AND WINTER TEMPERATURE SELECTION BY DESERT TORTOISES IN THE RINCON MOUNTAINS, ARIZONA. Draft manuscript to be submitted to the Journal of Wildlife Diseases: Jones, C. A., C. R. Schwalbe, and D. E. Swann.

LRH: C. A. Jones et al.

RRH: Effects of *M. agassizii* on home range and temperature selection

EFFECTS OF *MYCOPLASMA AGASSIZII* ON HOME RANGE OF AND WINTER TEMPERATURE SELECTION BY DESERT TORTOISES IN THE RINCON MOUNTAINS, ARIZONA

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ABSTRACT

Upper Respiratory Tract Disease (URTD), caused by the pathogens *Mycoplasma agassizii* and *M. testudineum*, has been documented in the desert tortoise (*Gopherus agassizii*) and gopher tortoise (*Gopherus polyphemus*). Although die-offs attributed to URTD led to the federal listing of the Mojave population of the desert tortoise as threatened, little is known about this disease in the Sonoran population of the desert tortoise. As part of a study assessing the presence of URTD across an urban gradient in Tucson, Arizona, USA, we used radiotelemetry to determine home range size and

temperature-sensing data loggers to determine winter temperature selection for desert tortoises at two sites in and near Saguaro National Park in the Rincon Mountains, Pima County, Arizona. We used enzyme-linked immunosorbent assay (ELISA) to detect antibodies indicating previous exposure to *M. agassizii*, examined the association between URTD status and minimum convex polygon (MCP), 50% kernel and 95% kernel (KHR) home range size, and examined the winter thermoregulatory behavior of asymptomatic and symptomatic desert tortoises. The percentage of seropositive tortoises in the Rincon Mountains (72.7%) is the highest reported for the Sonoran Desert; however, we found no significant difference between seropositive and seronegative tortoises for 100% MCP (P=0.55), 50% KHR (P=0.74), or 95% KHR (P=0.49) estimates. Two clinically ill desert tortoises exhibited daily activity during winter that resulted in increased carapace temperatures. Our results are consistent with monitoring results that suggest that while *M. agassizii* is widespread among tortoises in the Sonoran Desert, especially in urban areas, and worthy of continued study, URTD does not currently appear to be a major threat to this population.

Key works: *Gopherus agassizii*, home range, iButton, *Mycoplasma agassizii*, Sonoran Desert, temperature selection, thermoregulation, upper respiratory tract disease.

INTRODUCTION

Due to dramatic declines in the 1980s, the Mojave population of the desert tortoise (north and west of the Colorado River) was designated as threatened under the Endangered Species Act (ESA) in 1990 (USFWS, 1990); upper respiratory tract disease (URTD) was identified as a major causative agent in these declines (USFWS, 1994). Upper respiratory

tract disease has been studied extensively in the desert tortoise (*Gopherus agassizii*) in the Mojave Desert (Berry, 1997; Brown et al., 1999; Christopher et al., 2003; Jacobson et al., 1991, 1995; Lederle et al., 1997; Schumacher et al., 1997; Berry et al., 2006) and the gopher tortoise (*G. polyphemus*) in the southeastern United States (McLaughlin, 1997; Smith et al., 1998; Berish et al., 2000; Thomas and Blankenship, 2002; McCoy et al., 2007; Karlin, 2008). In contrast, little pathological information exists on free-ranging desert tortoise populations in the Sonoran Desert (Jacobson et al., 1991; Dickinson et al., 2002, 2005), which is not listed under the ESA. Clinical signs of URTD include intermittent serous, mucoid, or purulent nasal discharge, ocular discharge, palpebral edema, conjunctivitis, sunken eyes, and dullness of the skin and scutes (Jacobson et al., 1991; Schumacher et al., 1993; Brown et al., 1994). In 1994, *Mycoplasma agassizii* was found to be the putative cause of URTD in desert tortoises (Brown et al., 1994); and in gopher tortoises in 1999 (Brown et al., 1999); in 2004, *M. testudineum* was also identified as a cause of URTD in desert tortoises (Brown et al., 2004). This disease is highly contagious, transmitted by close contact between tortoises, and often clinically silent and long-lasting; some tortoises have remained infected for up to a year (Jacobson et al., 1995; McLaughlin, 1997; Schumacher et al., 1997).

Although considerable research has been conducted on URTD, the impact of this disease on tortoise on home range size or winter temperature selection has not been characterized. Researchers in the Sonoran Desert have observed symptomatic desert tortoises basking, foraging, and drinking during the traditional inactive season, November - March (S. Bailey, J. Jarchow, DVM, R. Repp, C. Schwalbe, E. Zylstra, unpubl. data).

Investigations into ectothermic thermoregulation caused by infection have been conducted with lizards (Kluger et al., 1975; Vaughn et al., 1974) as well as several turtle species (Monagas and Gatten, 1983; Swimmer, 2008).

As part of a larger study of URTD across an urban gradient in Greater Tucson, Arizona (Jones et al., 2008a), we used radiotelemetry to examine home range size, and small temperature data loggers to record winter temperature selection of adult desert tortoises in two locations in the Rincon Mountains, Pima County, Arizona. We sampled plasma from radio-telemetered tortoises to determine if they contained antibodies to *M. agassizii*, indicating previous exposure to URTD, when measured by an enzyme-linked immunosorbent assay (ELISA) (Schumacher et al., 1993). The goal of our study was to determine if URTD status, location, or sex affects home range size, and if symptomatic desert tortoises would exhibit thermoregulatory behavior that increased their environmental temperature during the winter in response to an active clinical infection.

MATERIALS AND METHODS

We studied tortoises at two established desert tortoise study sites in the Rincon Mountains, located east of Tucson, Arizona (Figure 1). The first site, Mother's Day Fire (MDF), is located entirely within the boundary of Saguaro National Park East (SNPE). Radiotelemetry and mark-recapture has been used since 1996 at this site to investigate effects of fire (Esque et al., 2003), population genetics (Edwards et al., 2004), reproductive ecology (Stitt, 2004), and nutritional ecology (Oftedal, 2007) of Sonoran desert tortoises. The second site, the Rocking K Ranch (RK), is approximately six km south of MDF and located along the SNPE boundary. Telemetry has been used at the RK

since 1999 to investigate potential changes in desert tortoise habitat use as a result of future development (Schwalbe et al., 2002), estimate density (Swann et al., 2002), and study population genetics (Edwards et al., 2004) and reproductive ecology (Stitt, 2004).

From June 2002 to June 2004, we searched for desert tortoises in both study sites. We examined each tortoise encountered for clinical signs of URTD (nasal discharge, ocular discharge, palpebral edema, and conjunctivitis), shell anomalies, and parasites, and to determine sex (Murray and Schwalbe, 1997). We weighed tortoises with a 1, 5, or 10-kg spring scale and measured the midline carapace length (MCL) with pottery calipers and a metal ruler to the nearest 1 mm (Christopher et al., 1997). We assigned each tortoise a unique number that was permanently notched into their marginal scutes with a triangular file following the standard notching system for Arizona (AIDTT, 2000) modified from Cagle (1939). In addition to the notches, we also assigned each tortoise an identification number which was applied to the fifth vertebral scute with correction fluid and black ink, then covered in gel epoxy to facilitate easy identification throughout the study (Murray and Schwalbe, 1997).

We affixed transmitters (AVM Instruments Co., Livermore, California) to the right front side of the carapace using five-minute gel epoxy, with the antenna threaded through rubber tubing to facilitate transmitter replacement (Boarman et al., 1998). Care was taken to not epoxy across scute seams where shell growth occurs. Up to 22 tortoises were located once every 7-12 days during the active season (March through October) and every 2-4 weeks during the inactive season (November through February) using a directional antenna and receiver; not all of these tortoises were part of the study at the

same time (Table 1). We used hand-held Global Positioning System (GPS) units (Garmin E-map, GPS III-plus, Geko201; Olathe, KS) to determine the location of each tortoise encountered as Universal Transverse Mercators (UTMs), using NAD 27 CONUS as the datum. To prevent the transfer of pathogens between tortoises, we wore fresh examination gloves for each tortoise and washed our hands and all equipment with veterinary disinfectant (chlorhexidine diacetate; AIDTT, 1996) after processing each tortoise. All tortoises were processed at the site of capture and released within one hour.

We programmed iButtons (Thermochron DS1921G iButton; Maxim Integrated Products, Inc., Sunnyvale, CA), small temperature data loggers (16 mm diameter, 4-g), to record the winter environmental temperature selection, not the body temperature, of desert tortoises at MDF and RK; the temperatures recorded the approximate carapace surface temperatures near the iButton. We programmed the data loggers to record temperatures (˚C) once every two hours, and affixed them using five-minute gel epoxy to the left fourth costal scute of telemetered tortoises prior to the expected onset of hibernation at the MDF site in winter 2002-3 (n = 9), and at both sites in winter 2003-4 (n = 9), and 2004-5 (n = 9). To determine if a tortoise emerged from its hibernacula, we epoxied data loggers to rocks and placed them in winter burrows of each tortoise equipped with a data logger to compare each tortoises's carapace temperature with their burrow temperatures. The iButtons were removed from the tortoise and burrow several weeks after emergence from hibernation, and downloaded using iButton-TMEX 32-Bit (Ver. 3.20; Dallas Semiconductor, Dallas, TX).

To determine if a tortoise had been exposed to *M. agassizii,* we collected ≤1 cc of blood via subcarapacial or brachial venipuncture from each telemetered tortoise for an ELISA to detect antibodies against *M. agassizii* (Schumacher et al., 1993). Blood samples were stored on ice in a lithium heparin buffer and centrifuged within 12 hours to separate the plasma. The plasma was stored at -20°C in a manual defrost freezer. The red blood cells were archived for future population genetics studies. In August 2004, we collected a second blood sample from seven tortoises to determine if the ELISA titer level for anti-*M. agassizii* antibodies changed during the study.

We shipped the plasma samples overnight on dry ice to the Mycoplasma Research Laboratory, College of Veterinary Medicine, University of Florida (Gainesville) for an ELISA which reveals if a tortoise has been exposed to *M. agassizii*. A positive result (titer ≥64) indicates that the tortoise has been previously exposed to *M. agassizii*. A negative result (titer <32) indicates that there are no detectable antibodies to *M. agassizii* in the plasma provided to the laboratory. A suspect result (titer 32-64) indicates that the antibody level is intermediate between positive and negative, and is considered inconclusive without retesting.

We performed all statistical analyses with JMP software (Ver. 4.0; SAS Institute, Inc.). We determined cumulative home range size of 22 radio-telemetered desert tortoises as 100% minimum convex polygon (MCP; White and Garrott, 1990), 50% fixed kernel and 95% fixed kernel home range (KHR) estimates (Worton, 1989), using the Animal Movement Extension in ArcView GIS (Ver. 3.3; Environmental System Research Institute, Redlands, California; Hooge et al., 1999). Due to the bimodal distribution of the

MCP and KHR sizes, we used natural-log transformations of home range data in our analyses (Ramsey and Schafer, 2002). We used ANOVA to determine if the number of observations for each tortoise was correlated with MCP, 50% and 95% KHR; because there was no correlation (P=0.27, P=0.81, and P=0.33 respectively), we excluded this variable from our models. We used ANOVA to examine the association between disease status, location, and sex on home range size, with natural-log transformed MCP, 50% KHR or 95% KHR as response variables, and ELISA results, location, and sex as explanatory variables (Zar, 1999). Home range estimates are presented as means plus or minus standard deviations. Because ELISA-suspect results are inconclusive without retesting, tortoises testing serosuspect for exposure to *M. agassizii* were excluded from our analyses.

We used SigmaPlot (Ver. 10.0; Systat Software, Inc. SigmaPlot for Windows) to graph each tortoise-burrow pair of temperatures recorded by the data loggers. We visually inspected each graph to compare the winter temperature selection for each tortoise and its hibernaculum, and determine if the tortoise exhibited winter activity.

RESULTS

We radio-tracked up to 13 desert tortoises (7 males, 6 females) at the MDF site, and up to nine tortoises (3 males, 6 females) at the RK site between June 2002 and April 2006 (Table 1). Individual tortoises were located 35 to 132 times ($\bar{x}$ = 69.6 ± 25.1). Two adult female tortoises (MDF 300 and RK565) made single, long distance movements; because each tortoise spent multiple days at these locations, data for these movements were considered part of the tortoises's home range, and included in our analyses.

Two adult male desert tortoises (MDF 221 and RK 103) were taken in for veterinary exams in November 2004 due to poor physical condition. Tortoise RK 103 was diagnosed with hypocalcemia and hypoalbuminemia likely due to poor nutrient intake and negative nitrogen balance at the end of the summer season. Tortoise MDF 221 was expressing clinical signs associated with URTD (clear nasal discharge, palpebral edema), and was seronegative for exposure to *M. agassizii* as shown by ELISA. One tortoise died during the study. Adult male MDF721 was submitted for necropsy at the Arizona Veterinary Diagnostic Lab on 14 Nov 2003; bacterial bronchopneumonia, a non-*Mycoplasma* infection in the lower respiratory tract was identified as cause of death (G. Bradley, DVM, pers. comm. 2003). The transmitter signals of seven tortoises were lost, presumably due to premature transmitter failure.

We collected blood samples from tortoises equipped with radio transmitters at the MDF (n = 13) and RK (n = 9) sites between August 2002 and August 2004. Antibodies to *M. agassizii*, as measured by ELISA, were present in 16 (72.7%) of the 22 samples. Five (6.4%) tortoises were seronegative, and 1 (4.5%) was serosuspect (Table 1). Of the 10 males, eight were seropositive, and two seronegative. Eight of the females were seropositive, three seronegative, and one serosuspect (Table 1).

To determine if anti-mycoplasma antibody titer levels change over time, seven tortoises sampled in 2002-3 were sampled a second time in 2004. Of these, five remained positive; one seroconverted from negative to positive, and one from suspect to negative (Table 2). The antibody titer level of two of the positive tortoises remained the same; however, the titer levels of the remaining three seropositive tortoises had decreased.

We estimated home range sizes from 1.4 to 65.1 ha (Table 1). ELISA results were not correlated with MCP ($F_{3,17}$ =2.37, P=0.55), 50% KHR ($F_{3,17}$ =0.75, P=0.74), or 95% KHR ($F_{3,17}$ =0.21, P=0.49; Table 3). Study site location was correlated with MCP home range size ($F_{3,17}$ =5.07, P=0.04); MCPs for tortoises at the RK ($\bar{x}$ =20.5±17.6 ha) were nearly twice as large as those at the MDF ($\bar{x}$ =10.7±14.7 ha); however, there was no correlation between location and either the 50% ($F_{3,17}$ =2.04, P=0.17) or 95% KHR ($F_{3,17}$ =0.10, P=0.75; Table 1). There was also no correlation between sex and MCP ($F_{3,17}$ =0.48, P=0.50), 50% ($F_{3,17}$ =0.00, P=1.0) or 95% KHR ($F_{3,17}$ =0.14, P=0.71) sizes.

We collected temperature data for four (two males, two females) desert tortoise-burrow pairs during winter 2002-3 and eight (4 males, 4 females) during 2004-5, for a total of 12 tortoise-burrow comparisons (Table 4). In 2003, only five of the nine iButton pairs were recovered. One of the data loggers was no longer attached to the tortoises when it emerged from hibernation; one data logger was not recovered from a burrow; and we lost the transmitter signals of two tortoises equipped with iButtons, presumably due to premature transmitter failure. Due to iButton program malfunction, no data were collected during winter in 2003-4. Eight of the nine data logger pairs were retrieved in 2005. We lost the transmitter signal for one tortoise equipped with an iButton, presumably due to premature transmitter failure.

Temperatures recorded by data loggers were clearly different for desert tortoises in or outside of their burrows (Table 4, Figure 2). Tortoise and burrow data logger temperatures for 5 of the 12 tortoises (males: MDF 339 and RK 414; females: MDF 298, 410, and 422) overlapped, indicating that these tortoises did not emerge from their winter

hibernacula until after April; telemetry data indicated that they did not emerge until the summer rainy season began in early July. Two of these tortoises (male MDF 339 and female MDF 410) shared a winter hibernaculum. Temperature data from three female tortoises (MDF 217, 300, and 722) demonstrated that they emerged from hibernation in February, behavior corroborated by telemetry data (Table 4). Telemetry data indicated that two male tortoises (MDF 221 and RK 108) with somewhat variable temperature recordings in spring were not winter active but used shallow, sun-exposed hibernacula.

Two male tortoises (MDF 721 and RK 103) exhibited daily thermoregulartory activity throughout the winter. Both were in poor physical condition.

DISCUSSION

Nearly 75% (16/22) of the desert tortoises in this study tested seropositive for exposure to *M. agassizii*. This percent of seropositivity, along with percentage of other tortoises tested near Tucson (Jones et al., 2008a), are the highest documented to date in the Sonoran Desert and suggest that the potential for infection may stem from captive tortoises in nearby Tucson or may be exacerbated by some type of interaction between urbanization such as noise, climate, or historic land use.

The antibody titer level of three of the five tortoises that remained seropositive decreased between sampling occasions. When initially tested, titer levels were high positives (256); at the time of their second test in 2004 they were moderate positives (128). These results were consistent with previous studies that have demonstrated that antibody titer levels may fluctuate over seasons and time; levels tend to be highest when a tortoise has been recently infected, and decrease when there has been a lapse from the

onset of infection (Schumacher et al., 1997). It is not surprising that one tortoise's titer level changed from negative to positive, as there is a very high percentage exposure to *M. agassizii* (72.7%) at these two sites. The seroconversion from suspect to negative provides further evidence that all tortoises with suspect results should be retested to achieve accurate detection of evidence of exposure to *M. agassizii*.

Both sample size and number of observations per tortoises have strong bearing on measurements of space use and home range size. Harris et al. (1990) suggested that at least two home range estimators should be used for each dataset. The estimated 100% MCP home range sizes (13.9±15.3 ha for seropositive tortoises, and 17.9±21.1 ha for seronegative tortoises) are similar to those from other studies in the Sonoran Desert where sample sizes of 3.1 to 93.4 ha (23.9±23.0 ha) were reported by Riedle et al. (2008), and data combined from six studies calculated home ranges of 14.3 ± 13.72 ha (Averill-Murray et al., 2002). Desert tortoises at the RK had larger MCP home ranges than those at MDF; yet there was no difference with either the 50% or 95% KHR. Studies have shown that home range size estimators are influenced by sample size; sample sizes of <100 underestimate MCP, and those <30 overestimate KHR (Bekoff and Mech, 1984; Seaman et al., 1999). Although we found no correlation between number of observations and the home range estimators used in our study, the tortoises at the RK were observed more frequently than those at MDF so the MCP for tortoises at the MDF may have been underestimated.

Home range size did not differ between seropositive and seronegative tortoises in our study, suggesting that seropositivity alone does not significantly affect movement. It

is possible that tortoises in the Sonoran Desert may have natural antibodies to *M. agassizii* that allow them to live with this pathogen and not become symptomatic unless other factors weaken their immune system (Jacobson et al., 1991; Hunter et al., 2008). Alternatively, if *M. agassizii* is introduced, as some researchers believe (Jacobson et al., 1991), then Sonoran desert tortoises may have natural immunity to other pathogens that may also be effective against *M. agassizii*, or that environmental factors in the Sonoran Desert allow natural resilience to it.

Monitoring tortoises in the Sonoran Desert over the past several decades has revealed few major die-offs of any kind, and none that appeared to be associated with URTD (Barrett, 1990; Dickinson et al., 2002, 2005; Johnson and Averill-Murray, 2004; Riedle and Averill-Murray, 2004; Jones et al., 2008a, b). The high mortality rates attributed to *M. agassizii* in desert tortoises in the Mojave and Colorado deserts do not seem to be occurring in the Sonoran Desert (Berry, 1997; Jacobson et al. 1991; USFWS, 1994).

In ectotherms, elevated body temperature results in increased metabolic activity, and one potential benefit is to enhance the immune system. Research on lizards (Vaughn et al., 1974; Kluger et al., 1975; Kluger, 1978) and frogs (Kluger, 1977; Woodhams et al., 2003) indicate that individuals using behavior to attain a febrile state in response to infection have higher rates of survivorship than individuals maintained at lower temperatures. Several studies have explored behavioral fever in response to infection in turtles. Monagas and Gatten (1983) introduced an *Aeromonous hydrophila* infections into eastern box turtles (*Terrapene carolina*), which resulted in mean elevation of body

temperature of 4.6˚C above non-infected turtles through basking. Rare basking behavior has also been exhibited by green turtles (*Chelonia mydas*) in Hawaii in response to high rates of fibropapillomatosis, benign tumors that primarily occur on the skin, eyes, and cloaca (Swimmer, 2008). Although our sample size of symptomatic tortoises in this study was small, the two male desert tortoises in this study that exhibited behavior that elevated their carapace temperature daily over the winter also presented clinical signs of illness. Although other researchers in the Sonoran Desert have observed clinically ill tortoises remaining active throughout the winter, our study is the first to quantify this activity using temperature data loggers.

We found that winter activity varies by individual, which is consistent with the results of other studies in which some tortoises remain in their hibernacula throughout the winter (inactive) season, while others exhibit activity on warmer winter days (Burge, 1977; Vaughan, 1984; Bailey et al., 1995; Martin, 1995; Nussear et al., 2007). While most tortoises remained in their hibernacula throughout the winter, three females emerged from hibernation in February, consistent with spring activity patterns observed in other Sonoran and Mojave desert tortoises (Burge, 1977; Huey, 1982; Vaughan, 1984; Burge et al., 1989; Bailey et al., 1995; Averill-Murray, 2002; Rautenstrauch et al., 2002). Female tortoises in the Sonoran Desert typically lay eggs every two years, and spring behavior including basking and foraging is probably necessary for reproducing females to develop eggs, which are typically deposited in June (Bailey et al., 1995; Averill-Murray et al., 2002; Stitt, 2004). Averill-Murray (2002) found a strong correlation between winter and spring rainfall and fecundity in female Sonoran desert tortoises. It is possible

that spring termination of hibernation by female tortoises may be a thermoregulatory and foraging strategy that leads to maximum egg development. Bailey et al. (1995) found that following wet winters male tortoises would emerge in the spring (April-May), but following dry winters males tended to stay in their hibernacula until onset of summer rains.

Our temperature data sample size and quality would have been improved by increasing the number of data loggers used in the surrounding environment and disabling the rollover function of the loggers. We attempted to program all iButtons to disable the rollover function which would allow data to be written over once the memory is full; however, all iButtons programmed in December 2003 did rollover and wrote over the recorded temperature data. The original data cannot be recovered once it is written over (K. Edmundson, Dallas Semiconductor, pers. comm. 2004). Data quality with iButtons can also be improved by using deflectors (i.e., aluminum foil) in burrows, which serve to decrease variation in temperature due to direct sunlight. One iButton placed in a west facing burrow recorded temperature fluctuations between 5 to 13˚C above the tortoises' carapace temperature on 57 occasions from December 2002–January 2003; the time and temperature combination corresponded with sunlight from the setting sun entering the burrow.

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Table 1. Number of observations, MCP, 50% and 95% KHR, and ELISA (positive = +; negative = -; suspect = S) and clinical sign (CS) results of desert tortoises in the Rincon Mountains, Pima County, Arizona, 2002-6.

ID#	Sex	MCL (mm)	URTD Status		Home Range Size (ha)				
			ELISA Results	CS	MCP	50% KHR	95% KHR	# Obs	Years in Study
Mother's Day Fire									
MDF 126	F	234	+	-	4.5	0.4	2.9	48	2002-4
MDF 204	F	208	+	-	3.9	0.2	1.9	51	2003-4
MDF 217	F	215	+	-	11.3	0.9	8.5	88	2004-6
MDF 221	M	243	-	-	7.6	0.5	4.7	132	2003-5
MDF 233	M	248	-	-	5.0	0.5	3.1	39	2003-5
MDF 300	F	204	-	-	55.2	4.6	16.0	49	2004-5
MDF 339	M	264	+	-	3.6	0.2	1.7	48	2002-4
MDF 390	M	259	+	-	4.2	0.8	6.1	68	2002-5
MDF 410	F	248	+	-	3.3	0.3	2.4	65	2002-4
MDF 422	M	269	+	-	11.9	0.4	3.0	97	2002-5
MDF 712	M	236	+	-	16.3	1.4	11.8	70	2002-4
MDF 721	M	219	+	2002-3	1.4	0.3	1.7	36	2002-3

Table 1. Continued.

ID#	Sex	MCL (mm)	URTD Status		Home Range Size (ha)				
			ELISA Results	CS	MCP	50% KHR	95% KHR	# Obs	Years in Study
Mean					10.7	0.9	5.3		
95% CI					1.3 - 20.0	0.1 - 1.6	2.4 - 8.2		
Rocking K Ranch									
RK 103	F	234	-	2004-5	8.4	0.4	4.2	108	2002-5
RK 404	M	271	+	-	13.3	0.4	1.4	83	2002-5
RK 414	M	240	+	-	10.7	1.5	9.8	90	2002-5
RK 459	F	240	+	2002-3	9.8	0.7	2.7	35	2002-3
RK 480	F	244	+	-	19.2	1.7	0.7	62	2002-4
RK 481	M	250	+	-	24.2	3.1	20.0	66	2002-4
RK 485	F	233	+	-	20.2	0.6	4.1	89	2002-4
RK 565	F	256	+	-	65.1	2.4	15.2	75	2002-4
RK 770	F	240	-	-	13.3	0.4	4.3	63	2002-4
Mean					20.5	1.2	6.9		
95% CI					7.0 - 34.0	0.5 - 2.0	1.8 - 12.1		

Table 2. ELISA results (positive = +, negative = —, suspect = S) from telemetered tortoises sampled in both 2002 and 2004.

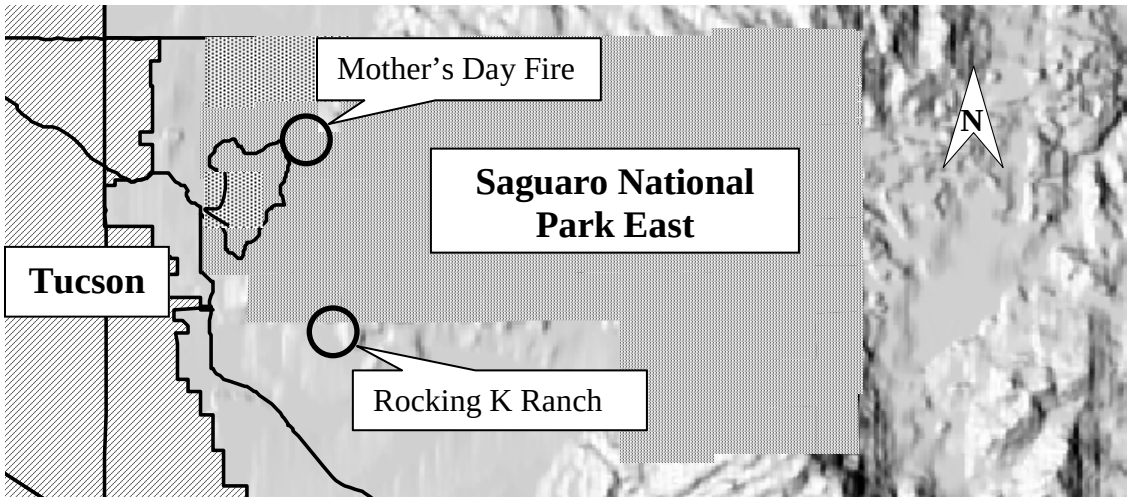
	2002		2004	
ID#	ELISA	Titer	ELISA	Titer
MDF 126	+	256	+	64
MDF 204	+	256	+	256
MDF 233	S	32	—	<32
MDF 339	+	128	+	128
MDF 410	+	128	+	128
MDF 422	+	256	+	128
MDF 712	—	<32	+	64

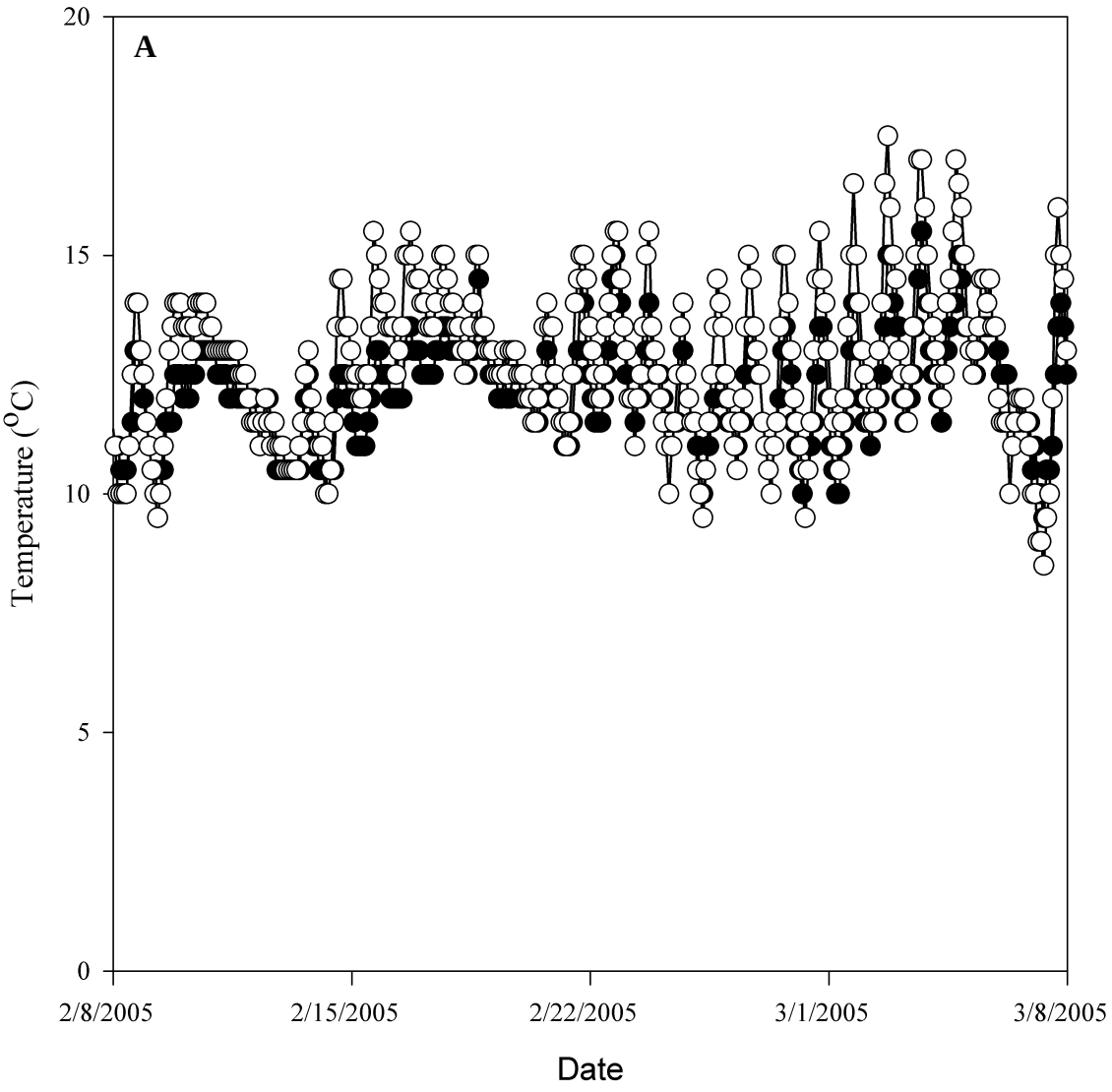
Table 3. Home range sizes for seropositive (ELISA +, *n* = 16) and seronegative (ELISA-, *n* = 5) tortoises from 2002 to 2006.

	ELISA +			ELISA -		
	$\bar{x}$		SD	$\bar{x}$		SD
100% MCP (ha)	13.9	±	15.3	17.9	±	21.1
95% KHR (ha)	5.9	±	0.9	6.5	±	1.8
50% KHR (ha)	0.9	±	5.7	1.3	±	5.4

Table 4. ELISA and clinical sign status and winter thermoregulatory behavior (as measured by activity) of desert tortoises equipped with temperature data loggers at two sites in the Rincon Mountains, Pima County, USA.

ID#	SEX	ELISA	CS	Dates	BEHAVIOR
MDF 721	M	+	-	2002-3	Daily Activity
RK 103	M	-	+	2004-5	Daily Activity
MDF 217	F	+	-	2004-5	Early Emergence
MDF 300	F	-	-	2004-5	Early Emergence
MDF 722	F	S	-	2004-5	Early Emergence
MDF 298	F	+	-	2002-3	Inactive
MDF 339	M	+	-	2002-3	Inactive
MDF 410	F	+	-	2002-3	Inactive
MDF 422	F	+	-	2004-5	Inactive
RK 414	M	+	-	2004-5	Inactive
MDF 221	M	-	-	2004-5	Shallow Burrow
RK 108	M	+	-	2004-5	Shallow Burrow


Mother's Day Fire
Saguaro National Park East
Tucson
Rocking K Ranch
N


A
20
15
10
5
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Temperature (°C)
2/8/2005
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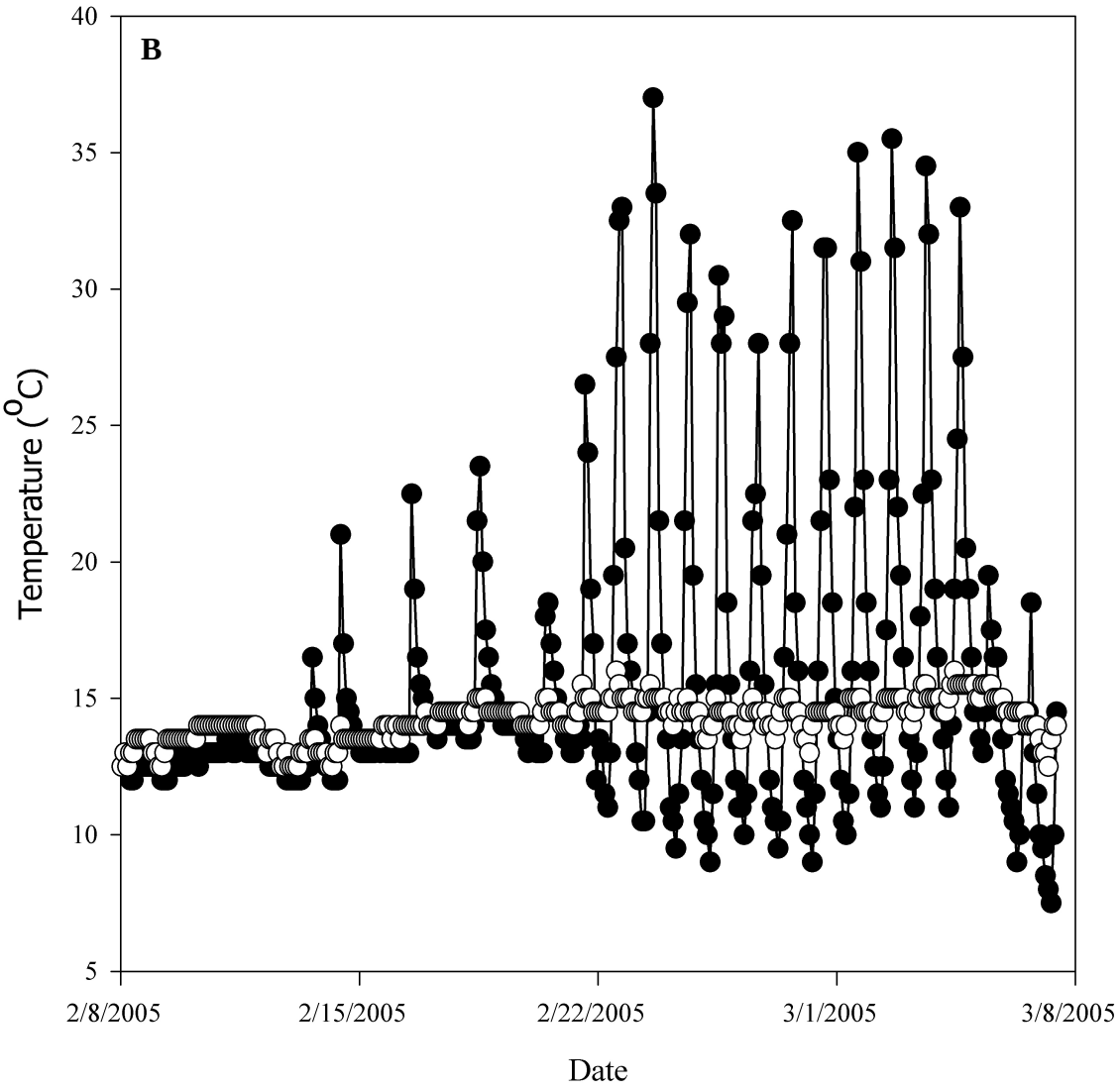
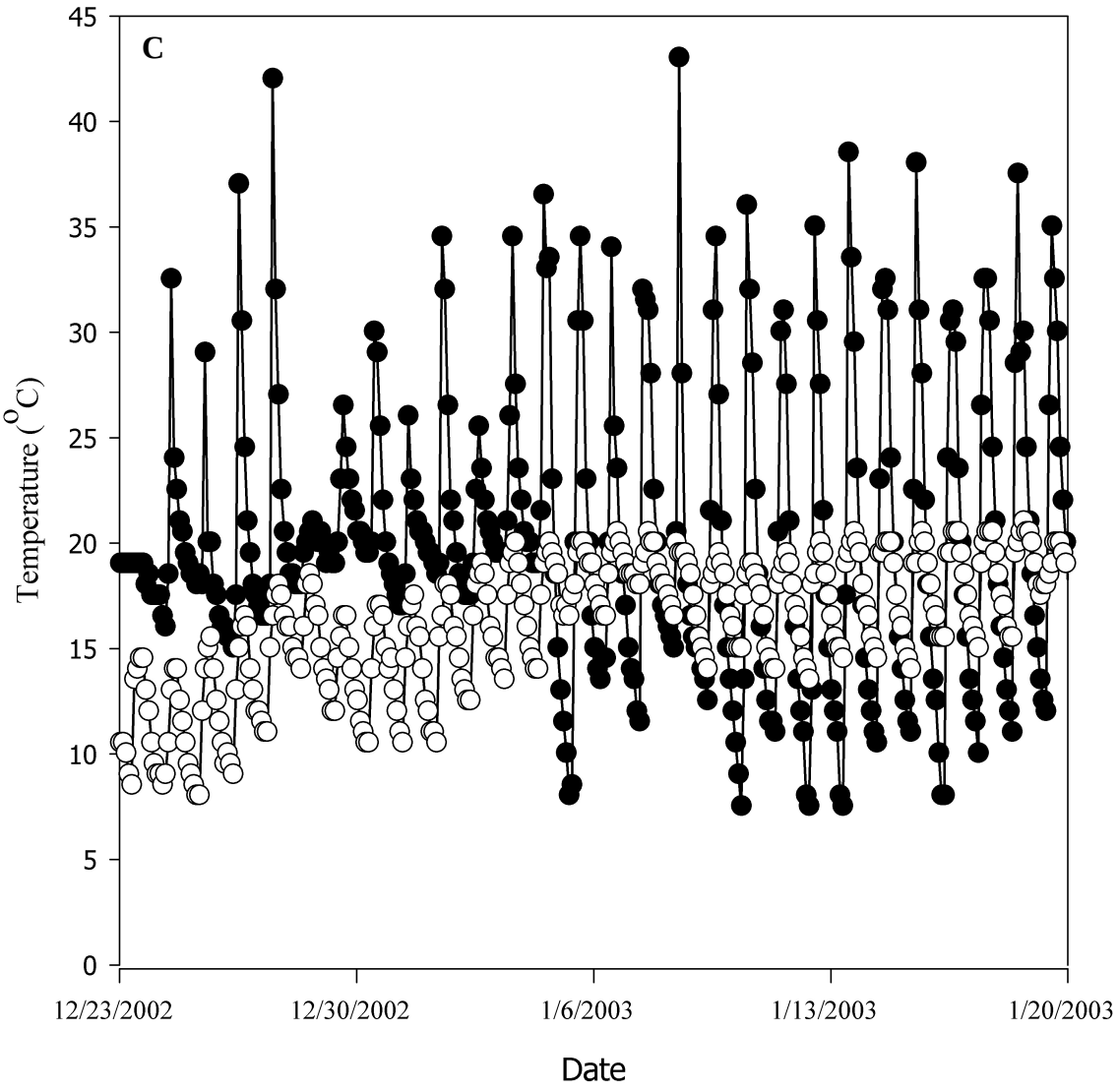
3/1/2005
3/8/2005
Date


B
40
35
30
25
20
15
10
5
Temperature (°C)
2/8/2005
2/15/2005
2/22/2005
3/1/2005
3/8/2005
Date


C
45
40
35
30
25
20
15
10
5
0
Temperature (°C)
12/23/2002
12/30/2002
1/6/2003
1/13/2003
1/20/2003
Date

LIST OF FIGURES

Figure 1. Study sites in and adjacent to the Saguaro National Park, Rincon Mountain District, Pima County, USA. Mother's Day Fire site is within the park, and the Rocking K Ranch is adjacent to the park, and spans the park south boundary.

Figure 2. One month of temperature data recorded to provide examples of a tortoise that did not emerge from hibernacula (A), a tortoise that terminated hibernation early (B), and a symptomatic desert tortoise that exhibited frequent winter activity (C). Data are presented as tortoise (filled circles) and burrow (open circles) temperatures.